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PREFACE

With the passing of the years, the *Annual Review of Biochemistry* tends to become more and more obese. We find that we cannot, with good grace, reduce its intake, nor can we contemplate with equanimity any process of fission. Beginning with the present volume and continuing through the sister Reviews, we are endeavoring to save a few pages hitherto devoted to the bibliographies. We trust that the shift to a two-column format will be acceptable and that the slightly smaller type size in the bibliographies will not be considered as an unpardonable assault upon the visual mechanisms of our colleagues in the sciences.

In still another respect this volume differs from its predecessors. The last few pages are given over to a Chapter Title Index and Chapter Author Index for Volumes 21 to 29 inclusive. This section will be continued as a running index in future volumes with listings from the oldest volumes deleted, as necessity may dictate. The publication of a third decennial index, as a separate volume, is not contemplated.

Again we wish to pay tribute to the many who have written for the *Review*. They have co-operated with us generously. They have given to the task an extraordinary amount of time and effort and have responded in good spirit and understanding to the many suggestions and requests that flowed out from the busy pens of the editorial staff.

We also express our very deep sorrow in the death of Hermann O. L. Fischer and of Kai Linderstrøm-Lang. The former, it will be noted, wrote the prefatory chapter for this present volume. This was the last of his many papers among which should be mentioned two earlier contributions to the *Annual Review of Biochemistry*—to Volume 11 with J. C. Sowden and to Volume 20 with D. L. MacDonald. Professor Linderstrøm-Lang, also a contributor to this *Review* (Volume 22, with Max Møller), had long ago accepted an invitation to write a prefatory chapter; it was his hope and ours that the article would be ready for publication within the next year or two. In the death of these men the world of science has lost two of its most dedicated disciples.

To our editorial assistants, and especially to Mrs. Betty Schink, we extend our very grateful thanks for their devoted help. We wish also to thank Dr. James P. Scannell who accomplished the very arduous task of preparing the subject index. Our printers, the George Banta Company, Inc., gave to us their complete co-operation and responded in fine spirit to our many demands upon their services.

F.W.A. G.M.
H.E.C. E.L.S.
F.S.D. E.E.S.
J.M.L. E.S.

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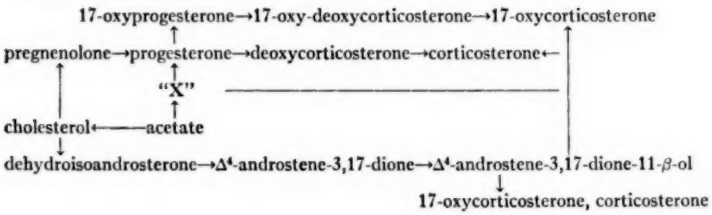

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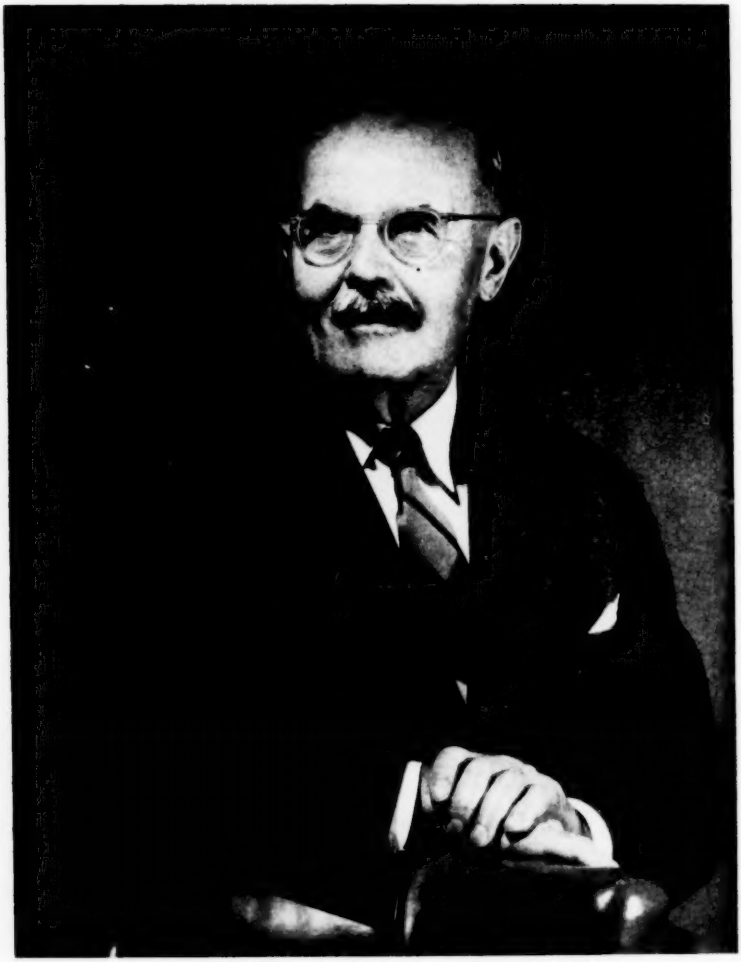

Hermann C. G. Finster.

PREFATORY CHAPTER

FIFTY YEARS "SYNTHETIKER" IN THE SERVICE OF BIOCHEMISTRY

By Hermann O. L. Fischer[1]

Department of Biochemistry, University of California, Berkeley, California

On the campus of the University of California at Berkeley, overlooking the beautiful San Francisco Bay, stands the Biochemistry and Virus Laboratory. The imposing building houses on its fourth floor, in a cedar-panelled room, the Emil Fischer Library. This is a medium-sized, modern reference library serving, in particular, the students and staff of the Departments of Biochemistry and Virology. It is built around the sizeable collection of scientific books of Emil Fischer. He started collecting books in the 1870's, and the oldest journal, *Berzelius' Jahresbericht der Chemie*, dates back to the year 1823. Old and new material in organic chemistry and biochemistry are well blended, and, by looking at the volumes which are for the most part in German and English, one can follow easily the evolution of biochemistry on the Continent, in England, and in this country.

This is a good place to remember the past, and I will try, within the limits of this prefatory notice, to give the reader an idea of what I have seen and learned in science during the long and turbulent period of human history through which I have lived.

My father, Emil Fischer, was Professor of Chemistry at the University of Würzburg in Bavaria when I was born in that pleasant and beautiful old town on the River Main as the first child of my parents in December, 1888. In Würzburg, my father experienced one of the most fruitful periods of his scientific life. He worked out the famous syntheses in the sugar group and the configurations of the pentoses and hexoses. In later days, he frequently told his family and friends that he would have lived a much more happy life had he remained in Würzburg instead of going to Berlin where administrative and social obligations deeply cut into his time for research. The period in Würzburg was also especially pleasant because the University allotted him sufficient funds to carry out his researches. Before that, he could pursue his scientific program only with the help of his father, Laurenz Fischer, a successful businessman in the Rhineland, who did not think very highly of scientific endeavor but dearly loved his son.

From Würzburg, the family moved to Berlin in 1892. Here my father, not yet forty years of age, succeeded to the Chair of August Wilhelm von Hofmann. He occupied the very spacious "Academic House" in Dorotheen-

[1] It is with deep regret that we record the death of Dr. Fischer—March 9, 1960. ——Ed.

strasse 10, which belonged to the Prussian Academy of Sciences, and directed from there the activities of the old Chemical Institute of Berlin University. By mere proximity, my two younger brothers and I came in contact very early with all facets of academic life and chemical research. We children were presented to many famous scientists of this period. I can remember particularly well the two-week stay of Sir William Ramsay in our house. He became a lifelong friend of my father and a most gracious host to myself during my later stay in England. Sir William was a brilliant speaker and gave, among others, a lecture on his discovery of the noble gases—argon, helium, krypton, and neon—before Emperor William II and his entourage in the main lecture room of the Chemical Institute. I still have in my collection of papers a postcard from Sir William announcing to his friend Fischer the discovery of argon. Events like these naturally stimulated the interest of the Fischer boys in the sciences.

In 1895, our family life received a terrible blow by the untimely passing of my beautiful, beloved mother. We three boys grew up in the somewhat drab household of our widowed father and early learned to be independent. Of great influence, however, were the conversations with our father at lunchtime. He had a great gift for explaining things in a simple and impressive way to students and children, frequently even using the form of old Rhinelandish peasant proverbs. (The family came from the left bank of the Rhine.)

Our school was an excellent "humanistic" gymnasium. Latin, Greek, history, and German were taught very well, mathematics somewhat, and science very little. Praiseworthy in the school was the high caliber of the teaching personnel and the truly liberal spirit in conducting the education of the boys. In the subject of religion, for instance, liberal historical books were recommended and used which hardly would find the approval of school authorities in some western democracies nowadays.

When it came to choosing a profession, father gave us full freedom. His own example, however, and the proximity of the new, big Chemical Institute, teeming with activity, pointed to science and medicine. My brothers chose medicine, and I decided to become a chemist.

When this decision was made and school finished, my father, conscious of the international aspect of science and deeply impressed with the philosophy of the English-speaking people, sent me straight from the German school bench to Cambridge University in England. The England of 1907 exerted an overwhelming influence on a pliable German youth of eighteen; I learned to know and to like the English and their way of life. Many years later, the decision to immigrate to Canada was made much easier by these experiences. Circumstances at Cambridge were extremely pleasant, and, although I did not learn much chemistry, I became very familiar with the workings of a great University and the ways of thinking and living of students and dons. My father's friends, particularly Sir William Ramsay and Professor H. E. Armstrong, extended the kindest hospitality to me in London, Lewisham, and Scotland. After one happy year in England, I returned to

Germany to serve my military year in the little town of Lüneburg and then returned to the study of chemistry, first in Berlin and then in Jena. The decision to study in Jena was reached only on second thought after my father found out that, in another university, where I originally wanted to go, the habits in the Chemical Institute were somewhat too alcoholic. The choice could not have been better, however, since, in the person of Professor Ludwig Knorr, I found a most brilliant and understanding mentor. At this time in his laboratory, part of his students worked on morphine and related substances and the other half on keto-enol tautomerism of the β-diketones. I was assigned to prepare the enol form of acetyl-acetone and, after some initial difficulties, succeeded fairly well. The scientific atmosphere in the Chemical Institute was very good, and we learned very much in chemistry and physics from Professors Knorr, Paul Rabe, and Wilhelm Schneider. With my doctor's degree acquired, I went off to Berlin to my father's laboratory to continue research under his guidance. This was the year 1912, and I consider the short period from 1912 to 1914 the happiest time of my life. This period has always served me as a kind of yardstick in scientific matters. The Institute, under the direction of Emil Fischer, was then one of the great scientific centers in Europe. It still housed all the branches of chemistry, except physical chemistry, in its vast halls and numerous special rooms, and one found an expert in almost every field of chemistry among the people working there. To mention only a few names, there were Professors Gabriel, Diels, Leuchs, Wilhelm Traube, Tiede, and Franz Fischer representing organic chemistry, inorganic chemistry and also radiochemistry in the persons of Otto Hahn and Lise Meitner. Of course, dominating the organic division, was my father himself. At twelve o'clock every day, having given his big lecture for 550 students, he came to the organic halls and talked to everybody, student and staff member alike. Every scientific subject could be brought before him. He always encouraged; he never discouraged, and he even sometimes gave an originally foolish proposition an intelligent twist so that it developed into a good and interesting paper.

In Hall #1 and in his private laboratory he had seven to eight "*Doctoranden*" and private assistants working for him. He never took on more, and he supervised their research very much in detail. When a co-worker was stuck with a problem, he was summoned to the private laboratory and the Chief's special bench of reagents. My father had the great gift of finding out by simple and numerous test-tube experiments the cause of the trouble, and he did not hesitate to excuse the co-worker in question, if he deserved it, with a negative compliment. He sat on such occasions on a high oaken laboratory stool inherited from A. W. von Hofmann. This stool was later appropriated by myself and brought, through all my many moves, finally to Berkeley. Here the members of my little group and I sit on it when stuck with our problems, to get inspiration—from below. Another great source of stimulation for the more advanced members of the Institute was his seminar (Colloquium) held weekly with a group of about sixty people. Here everyone

could speak his mind, and the Chief also commented very freely. Many new ideas came forward from this exchange of opinions.

For the students, the big attractions were the main lectures on inorganic and organic chemistry. The lectures were beautifully prepared by his faithful preparator of many years, Johannes Wetzel, and went on with the precision of a stage "performance," which was also what the students called them. I was privileged to hear both lectures, and I still remember with delight when the reaction between red mercury oxide and white hydrochloric acid was carried out and the sublimate appeared on the cool side of the retort. These chemical steps were carried out to the Professor's recitation of the quotation from Goethe's Faust:

> Da ward ein roter Leu, ein kühner Freier,
> Im lauen Bad der Lilie vermählt
> Und beide dann mit offnem Flammenfeuer
> Aus einem Brautgemach ins andere gequält.
> Erschien darauf mit bunten Farben
> Die junge Königin im Glas,
> Hier war die Arzenei, die Patienten starben,
> Und niemand fragte: wer genas?

In this way he exercised a marvellous influence on his audience and managed to attach to many items, which might have slipped the mind of the hearer, some interesting poetic or historical allusion which sharply clawed itself into the memory. None of his school achieved an equal mastery of speech, but many used his technique with great advantage for their students.

By 1912 he had relinquished working on the great fields of carbohydrates, proteins, purines, and pyrimidines. There was still some sugar work being done, but his main interest had turned to the depsides and tannins. His most important co-workers at this period were Burckhart Helferich, Max Bergmann, Karl Freudenberg, K. Zach, and Kurt Hoesch, who later became his biographer. Myself, freshly arrived from Jena, was also assigned a depside problem, namely, the synthesis of the naturally occurring didepside, lecanoric acid. After some initial difficulties and occasional frowning by the Chief, I managed to prepare the key substance of the syntheses, dicarbomethoxy-orsellinic acid chloride, and to carry out synthesis of both para- and ortho-di-orsellinic acid; after so doing, I was admitted into the chemical community. My neighbor on the chemical bench during these difficult months was a tall, friendly, English-speaking chemist who gave me excellent advice on the handling of acid chlorides. He later became well-known in this country as Professor Hans T. Clarke of the Biochemistry Department of Columbia University.

A few years later, my father's extremely competent co-worker, Ernst Pfähler, discovered with him the migration of the fatty acid residues in glycerides along the glycerol chain and also postulated the formation of ring-form compounds as intermediates. This idea of acyl migration in alkaline medium exercised a great influence upon our thinking and was many years

later followed by the discovery, by Erich Baer in Toronto, of the migration of the phosphoryl residue under acid conditions.

While at this time the other sciences and chemistry were in full bloom in Berlin, the German capital was also graced by a spectacular development in the arts. The concerts in the "Philharmonie" were of the first order; the Royal Opera and the Komische Opera were excellent; the representation of the Ibsen plays in the Lessing Theater were very good, but, above all, there was an unheard of splendor on the stages directed by Max Reinhardt, starting with Shakespeare's *Midsummer Night's Dream*, culminating in the Royal Plays of Shakespeare, and ending with Goethe's *Faust*, Parts I and II. I attended a performance of Part II in the presence of the German Chancellor, von Bethmann-Hollweg, and the performance was such a powerful interpretation of the great philosophical work that the audience left the theater filled with deep appreciation and understanding. These were golden times but all too short, because, in 1914, the first World War destroyed completely this wonderful atmosphere.

To come back again to my father's work, I would like to mention experiments he carried out with his friend, Professor Kohlrausch, president of the Physikalisch-Chemische Reichsanstalt; the idea was that of transforming elements into each other. The two scientists felt that the transformation could be achieved if one could concentrate colossal amounts of energy in small quantities of matter. They treated hydrogen with cathode rays and hoped to find, spectro-analytically, total or partial transformation of the hydrogen. The experiments were negative, which explains why they never published anything about it. The investigation is comprehensively described, however, in Emil Fischer's autobiography "Aus Meinen Leben."

At the beginning of 1914, my personal plans began to crystallize rapidly. I had some experience in organic chemistry and the intense desire now to study biology and medicine with the idea that my hard-earned chemical tools might be useful. I had always felt that organic chemistry was the first step—or the springboard—in elucidating the much more complicated circumstances presented by the living cell; I planned to join my two brothers who already had the special and extensive training of medical men. We also felt at this time that biochemical problems were much too complex to be worked on by only one person, and so, without knowing the present-day word, we planned to form a "team." This nice plan was rudely interrupted by the outbreak of World War I. I had to go to the front in France, and my brothers joined the military medical service. Both of them died in the line of duty, and when, after four and one-half years, the war ended, it was impossible to come back to our ambitious plan. So all my life I have essentially remained an organic chemist with a strong predilection for synthesizing compounds useful for biological or biochemical purposes. When my fellow chemists reunited in the Chemical Institute in Berlin after the war, we were fortunate in that my father was still alive and still directing research work in the traditionally correct way. It was a great effort for me, and presumably for

the other younger men as well, to make up for the lost years, relearn all that we had forgotten, and bring ourselves up-to-date in the sciences. But, owing to the example of the great master, our efforts met with some success. Unfortunately, we lost him already in July, 1919.

Being left alone without a family I was fortunate to find, in my wife Ruth, in 1922, an understanding and devoted partner for life. We have two sons and a daughter.

Only after the stabilization of the German mark in 1924 did the Institute, under the guidance of Professor Hermann Schlenk, gradually return to its normal functioning. I was appointed Assistant Professor in the Institute, thus giving me the opportunity to participate in general instruction in the organic division and to continue some of the researches I had started before the war. I am also obligated to Professor Hermann Leuchs, my immediate superior, for his many valuable suggestions and much sound advice. I eagerly took part in the instruction in organic experiments and gave a small lecture with experiments, which attracted some students from the organic division and the medical school.

In the research field, of course, it was at this time just as difficult for a beginner to make a reputation as it is now. But it always helps if the work is the object of some scientific controversy, provided the younger person proves to be right. This was true with regard to the constitution and configuration of quinic acid, a substance which is rather widely distributed in the plant kingdom. Professor Paul Karrer published a formula for this acid which, at first, seemed right to us. So our little group was thrown into confusion, because we had already invested several years of hard work on this problem and believed for a short while that our efforts were in vain. Upon closer investigation, however, we found that a formula different from Karrer's represented the true constitution of the acid. This was proven conclusively, and we were able to elucidate the constitution of chlorogenic acid, the well-known depside of quinic acid, and caffeic acid as well. This depside carries the caffeine in the coffee bean and determines largely the taste of coffee upon roasting. My trusted co-worker in this difficult work was Dr. Gerda Dangschat, née Anger. She had written her doctoral thesis on the synthesis of linamarin under my father, later married Dr. Paul Dangschat—also a "*Doctorand*" of Emil Fischer—and, when her family was sufficiently grown up, returned to the laboratory under my direction.

Parallel to the work on the plant acids, was our work on the trioses, dihydroxyacetone and glyceraldehyde. The first work was done by Dr. Carl Taube, now in Leverkusen, Bayerwerk, and then Dr. Erich Baer, of Berlin, who was to co-operate with me for the long and fruitful period of 27 years. Dr. Baer's first discovery was the rearrangement of glyceraldehyde into dihydroxyacetone by warm pyridine, a method now widely used to transform aldoses into ketoses. By the joint efforts of Lucy Ahlström, Hans Richter, and Fritz Kröhnke, we gained full understanding of this small, but tricky, field. Fritz Kröhnke was a distant cousin of mine and became a lifelong friend of our family. He was very successful in his scientific career and

is now professor of organic chemistry at the Justus-Liebig-University in Giessen, Germany.

The real reward for our work was soon to come. This was a time when, according to the fermentation theories of Neuberg, methylglyoxal was the key substance in the breakdown of sugar in fermentation and glycolysis. I could not believe that a substance, which is not fermentable itself, could be the central compound of fermentation; therefore, remembering the classical work of Harden & Young, we set out to prepare glyceraldehyde-3-phosphate (racemic), hoping that with three carbon atoms it would be the long-sought intermediate of fermentation. The synthesis was not easy, but we finally succeeded, particularly by using the benzyl residue as a blocking group. It can be easily introduced and, under very mild conditions, removed by catalytic hydrogenation. The method had been discovered by Otto Wolfes in studies on the chemistry of morphine and then applied by Karl Freudenberg to sugar problems. For our purposes, it was just the right thing, and, in the spring of 1932, I could bring to my friend, Otto Warburg, the first one and a half grams of the crystalline calcium salt of D,L-glyceraldehyde-3-phosphoric acid. Indeed, Dr. C. V. Smythe and Miss Gerischer found, in Warburg's laboratory, that the substance was fermentable by undiluted Lebedew juice to 50 per cent, which is perfectly understandable since, at this time, I could produce only the racemic form of the compound. Otto Warburg later carried out some of his classical work on this substance and even has done me the honor to call it "Fischer ester." At the time, however, Gustaf Embden and, later, Otto Meyerhof utilized the glyceraldehyde-3-phosphate for the development of their now famous scheme of alcoholic fermentation and glycolysis. Although I was soon to change my academic affiliation from the University of Berlin to the University of Basle, this most pleasant and rewarding cooperation with Meyerhof in Heidelberg was to continue.

It was by now 1932, and I was not totally blind to the threatening political developments in Central Europe. When an invitation came to join the staff of the University of Basle in Switzerland, I was glad to accept it. Very soon I could count Professor Hans Rupe, the Professor of Organic Chemistry at Basle, as one of my best friends. A special delight for my wife and myself was also the presence of our old and trusted friend, Dr. Oscar Schulthess and his wife, Paula. In many other respects, Basle was a delightful choice; away from the postwar turmoil of Germany, we rediscovered the placidity of "normal" European times as our parents and grandparents had known it. The scientific chemical atmosphere of the town was first-class because of the high standards of the Swiss chemical industry, and our children were well taken care of by the excellent and progressive Swiss schools.

Work soon started in the Institute at Totengässlein 3, close to the marketplace of the old town. Swiss students flocked in, and we had a lively and active group pouncing on various organic problems. The work on trioses and tetroses was continued. Erich Baer was able to prepare pure D- and L-glyceraldehydes and to work out his interesting synthesis of D-fructose and L-sorbose by condensation of dihydroxyacetone and D-glyceraldehyde under

the influence of mild alkali. D(+)-Acetone glycerol and its antipode, L(−)-acetone glycerol, were produced and used for the synthesis of optically active glycerides and glycero-phosphates. This was the occasion when the L-configuration of the natural α-glycero-phosphoric acid was proven conclusively, a result which, in turn, had its influence on the Embden-Meyerhof scheme. On the whole, the co-operation with Otto Meyerhof in Heidelberg proved to be most cordial and fertile. We travelled several times to Heidelberg to bring him new preparations of organic phosphates, and he, in turn, delegated his co-worker, Dr. W. Kiessling, to Basle to study chemical methods of phosphorylation. Parallel with this work, the elucidation of the configuration of quinic acid and shikimic acid proceeded in the hands of Dr. Gerda Dangschat and led to results that demonstrated fully the configurational connection of these acids with glucose. Of course, at this time we had no idea of the biochemical pre-eminence that shikimic acid would later attain. In about 1950, in Berkeley, I was able to provide Dr. Roger Stanier with a sample of shikimic acid, which he delivered to Bernard D. Davis. Davis, as it is well-known, discovered that, in bacterial metabolism, shikimic acid is one important intermediate in the formation of the aromatic amino acids.

Again in 1936, the climate in Basle, scientifically so favorable, was clouded by political considerations. It was only too clear that war was coming, and I did not want my two sons, who were approaching military age, to serve in an army whose ideals did not conform with those of the family. When, on a lecture trip to North America, I caught the eye of Sir Frederick Banting, who, together with Dr. Charles Best, had discovered insulin, I was only too glad to accept the invitation of these two men and of President Cody to join the staff of the University of Toronto.

On the same lecture trip, we saw Dr. and Mrs. H. T. Clarke in New York and had our first experience of American hospitality. They recommended us to the H. B. Vickerys at Yale University, and there we were treated in the same kind manner, so that we saw all the things to be seen in New Haven, met the chemists, and were put, with great honors, on the evening train to Washington. In Washington, we were the guests of Claude S. Hudson, the famous sugar chemist. I will never forget this meeting. It was just the period when he had worked out with Jackson the periodate method of splitting pryanosides and furanosides, thus proving the ring span of the glycosides in a most elegant manner. He brought me to his laboratory and showed me a big table where innumerable strontium salts of the dicarboxylic acids resulting from the oxidation of the dialdehydes were exhibited. The discussion of all these remarkable things was vivacious and even turbulent. In the evening we went to his apartment and spent a good deal of the night discussing his annotated volumes of *Emil Fischer: Kohlenhydrate und Fermente.* "Isn't it miraculous," he said, "so much work and so little wrong?" On this strong personal and scientific foundation, a lifelong great friendship developed. To give a visible sign of my admiration of him and his work, and also to express the thought of many colleagues—namely, that in sugar chemistry he was

really the successor of Emil Fischer—I gave him my father's golden pocket watch with a long, old-fashioned, golden chain on it. Claude used to wear watch and chain, as he said, "on state occasions," and never tired of pointing out their significance to questioners.

Hudson was also the spiritual focus of the "Starch Round Table," an event generously organized every year by the Corn Products Research Foundation. When he was present, there was never a boring moment, and it was remarkable with what speed and perspicacity he could discuss the most involved theoretical configurations, not to mention the sharpness and velocity with which he could fire amusing jokes and stories at the assembly. Besides his numerous famous publications, he did a very great service to carbohydrate chemistry when he founded, together with a circle of like-minded friends and under the auspices of the Academic Press, *The Advances in Carbohydrate Chemistry*, of which he was the first editor. His very able successor in the editorship is now Professor M. L. Wolfrom of Ohio State University. Understandably, I was very eager to get Hudson's approval of papers I sent to the journals, but he doled out praise, even to his friends, very rarely and almost economically. Only once, did I manage to extract a direct compliment from him: "Hermannol, this is really a good paper!" It was the conversion of D(+)-acetone glycerol into L(−)-acetone glycerol, its optical antipode, by stepwise exchange of substituents. The paper proved, in a new way, the enantiomorphic position of the substituents, the tricky experimental details having been worked out by Erich Baer.

In the fall of 1937 we reluctantly left Basle, lock, stock, and barrel, Fischers, assistants, private laboratory, and library, and arrived in Toronto, at the Banting Institute of the University of Toronto. In the Institute, space was provided by the generosity of the Department of Pathological Chemistry, headed by Professor Andrew Hunter, who, in the course of the years, became one of my best friends in Canada. He was always ready to help with scientific and personal advice. Another of the helpful friends of Dr. Baer and myself was Dr. C. C. Lucas of the Banting-Best Department, who not only did his very best in adjusting us to Canadian thoughts and habits, but also corrected, or even rewrote, our first publications to make them acceptable for the *Journal of Biological Chemistry*.

In the scientific field, my two trusted assistants, Dr. Erich Baer and Dr. Jean M. Grosheintz, with the help of some Canadian students—notably Dr. Leon Rubin who worked on the synthesis of selachyl alcohol—busily continued the work on trioses, glycerol derivatives, optically active glycerides, glycerol phosphates, and glycerol ethers and were supported by Dr. Banting in the most helpful way by gifts of natural products from his many physiological investigations. Besides this, Dr. Baer devised a very useful modification of the oxidation of glycols with lead tetraacetate in water. We were well set in our scientific endeavors when, in 1939, the Second World War actually broke out. I shall always have the greatest gratitude to the Canadian government and the University of Toronto that all through the war years we

were enabled to continue our work undisturbed and even aided by the government. The scientific vacuum generally created by warlike events was completely avoided, and we could continue during and after the war with full efficiency.

During the second year of the war, Canada, the University of Toronto, and myself suffered a catastrophic and irreparable loss: Dr. Frederick Banting, in an effort to take a newly designed pressure suit for crash divers to England, perished in an airplane accident on the Newfoundland coast. I shall forever preserve the deepest gratitude and admiration for this great scientist and truly magnanimous and altruistic man. The reins of the Department for Medical Research were very ably taken up by his friend, Dr. Charles Best. He was, for my little group and myself, a friendly and very understanding protector in wartime and an excellent department chairman.

Shortly before the outbreak of the war, our group was joined by Dr. John C. Sowden of Brantford, Ontario, who had his doctorate from M. L. Wolfrom's laboratory in Ohio State University. He proved to be a first-class sugar chemist and developed in my laboratory, with great talent, the condensation of aldoses with nitromethane. This represented a most useful and versatile procedure for lengthening the carbon chain of a sugar and for preparing numerous nitro and amino derivatives. Dr. Sowden has described these activities in an excellent review article in the *Advances in Carbohydrate Chemistry*. He is now chairman of the Department of Chemistry of the George Washington University in St. Louis, Missouri.

Dr. Grosheintz succeeded in developing an especially elegant variant of this procedure, which finally led to the cyclization of glucose into myo-inositol by way of a 6-nitro-6-deoxy-D-glucose or the corresponding L-idose derivative. When the cyclization was published in 1948, I had the special satisfaction of learning in a letter from my friend, Professor B. Helferich, that he had already tried the cyclization in my father's laboratory before the first war.

Dr. Henry A. Lardy, who, with his young wife, came to Toronto for one year in 1941,was interested in the chemical synthesis of biologically interesting organic phosphates. He came, like other Wisconsinites later, on the advice of my dear friend, Karl Paul Link. Lardy was the most pleasant and efficient co-worker one could imagine, and the twelve months he spent in our laboratory produced not only a number of good papers but also a lasting friendship. Henry was most successful in his later career and is now one of the directors of the Enzyme Institute at the University of Wisconsin in Madison.

While we were busy in Toronto with the cyclization of hexoses to inositols, on the other side of the ocean, Dr. Gerda Dangschat, under plans agreed upon previously, worked on other facets of inositol chemistry. She succeeded in oxidizing derivatives of the natural conduritol to muco- and allo-inositol, which at the time were new isomers of inositol. Later she proved the configuration of myo-inositol by oxidizing it via some intermediates to D, L-idosaccharic acid, a result which shortly thereafter was confirmed by

Th. Posternak. Her achievement is the more praiseworthy because it was done under the extremely difficult conditions of wartime Berlin.

Years later, the work on optically active glycerol derivatives was independently continued by my friend and former co-worker, Dr. Erich Baer, who is now head of the sub-department of organic chemistry in the Banting-Best Department for Medical Research at the University of Toronto. His great synthetic art has succeeded in producing various lecithins and cephalins, proving not only the configuration of these important compounds but also providing preparations of much higher purity than ever could be obtained from natural sources. His discovery of the migration of the phosphoryl residues along the glycerol chain in an acid medium led to most important consequences in the whole field of organic phosphates. Right after the war, our group in Toronto was joined by Dr. Beat Iselin who succeeded in synthesizing myo-inositol-2-phosphoric acid for the first time and is now working in the scientific department of CIBA in Basle, Switzerland.

Despite the excellence of the scientific and social climate in Toronto, there was no doubt that Mother Nature's climate was decidedly inclement. So, when the great opportunity came to move to Berkeley, I was only too glad to accept, particularly since the California weather promised to be much more favorable for my wife's health. I always will be deeply grateful to Dr. Wendell M. Stanley and the University of California for accepting me, in 1948, in spite of my age, as a member of the new Department of Biochemistry.

The new department was really a wonderful attraction. Dr. Stanley went about assembling an excellent staff of younger men, each one representing one special field of biochemistry or virology, and, when the group was welded into a working unit, it constituted a really powerful instrument to elucidate the many difficult and fundamental questions that the two disciplines embraced. For electron microscopy, he engaged Professor Robley C. Williams, an acknowledged authority in the field; for virology, C. Arthur Knight and, later, H. Fraenkel-Conrat, Gunther Stent, and Arthur Pardee; for physical chemistry, his old associate, Howard K. Schachman; for microbiochemistry, P. Kirk; for nucleic acid chemistry, Charles Dekker; for protein chemistry, Fred. Carpenter; for enzymes, J. B. Neilands; and for sugars and fats, D. L. MacDonald and myself. Besides these, there were, in close proximity, the notable members of the Department of Plant Biochemistry, H. A. Barker, W. Z. Hassid, P. Stumpf, and C. Delwiche. Until the new building was finished, the different members of these departments were scattered all over the campus but assembled in 1952 in the new building. Dr. Stanley invested an enormous amount of energy and technical and organizational ability into the planning and construction of what is now the Biochemistry and Virus Laboratory. The result justified the effort: great discoveries, like the splitting of the tobacco mosaic virus into protein and nucleic acid, and its reconstitution, came out of the new building as well as a stream of high quality publications. As for the physical plant itself, it is certainly one of the best planned, best arranged, and well organized buildings for biochemistry on

the whole continent. Time and again, I have heard visiting biochemists and other scientists express admiration for the fine technical and architectural features and immense practicality of the building.

Finally on October 9, 1952 (the date rings in my ears), the Biochemistry and Virus Laboratory was opened officially. It began with the dedication of the Emil Fischer Library on the fourth floor and with the observance of the 100th anniversary of the birth of my father. A particularly knowledgeable speaker, Vincent du Vigneaud, had come from New York to explain to the audience how, in older times, out of organic chemistry the modern and glittering science of biochemistry was born. He described, in an almost poetic way, how Emil Fischer was one of the great chemists of the past who used his mastery of organic chemistry for what we now call biochemical goals. He called Emil Fischer one of the fathers of modern biochemistry by virtue of his work in the field of carbohydrates, proteins, purines, and tannins. He described so well the essence of my father's work that it was moving, not only for me, but even for the more distant hearer. It was a great pleasure for me, after this wonderful introduction, to dedicate with a few simple words Emil Fischer's collection of books to the University of California. I also described the large collection of 9000 reference compounds representative of all the work of Emil Fischer. The collection, which had made possible numerous identifications of interesting compounds in past decades, is housed in our building. The index of this unique collection is rather unusual. To find a compound, one looks up the name in the *Collected Works of Emil Fischer*, finds the name of the pertinent co-worker, and subsequently seeks out an old cigar box with the name of the co-worker and his complete set of preparations. After my words of dedication, Dr. Stanley, in his capacity as Chairman of the Biochemistry Department and Director of the Virus Laboratory, accepted officially the Library and the bronze bust of Emil Fischer, well placed in the center of the beautiful room. The next day was filled by a most successful symposium on biochemistry and viruses in the International House auditorium, but lack of space limits me to a discussion of only two of the impressive presentations. There was again Dr. du Vigneaud who described his own work on protein hormones, with special reference to hormones of the posterior pituitary gland, for which he was later to receive the Nobel Prize. The other paper was given by my dear friend, Karl Paul Link, who, this time, was persuaded to attend a scientific meeting as a special favor to his friends, Wendell Stanley and "Hermannol." He most dramatically described the evolution of the anti-coagulant action of the dicumarins, starting from the elimination of the spoiled clover disease of the Wisconsin cattle to the death of millions of rats in continental America and Hawaii. The evening of the great day closed with a spirited banquet in the Hotel Claremont, Berkeley, under the toastmastership of President Robert Gordon Sproul of the University and with greetings from federal authorities and scientific bodies all over the world.

It was very fortunate that, at its inception, both the Virus Laboratory and the Department of Biochemistry remained for some time under the

leadership of Dr. Stanley. Later, under the pressure of expanding teaching and research activities, it seemed advisable that Dr. Stanley be relieved of some of his duties. So it fell to by far the oldest member of the whole group to substitute for him in the chairmanship of Biochemistry. This I did faithfully from 1953 to 1956, the date of my retirement. I also gave the lectures on carbohydrates and lipids in alternate years and tried to conduct the department according to the intentions of its founder.

With regard to my own researches, I relied mainly on the expert help of Dr. Donald L. MacDonald and Dr. Clinton E. Ballou. Donald L. MacDonald was associated with me from his student days in Toronto and faithfully followed me to Berkeley where he became an assistant professor. Many a good paper on sugar disulfones, sugar dialdehydes, inositol derivatives, tetrose phosphates (this one in co-operation with Clinton Ballou), and other compounds came from his laboratory bench and desk. He is presently engaged in pure research, working in the carbohydrate laboratory headed by that fine gentleman and worthy successor to Claude S. Hudson, Dr. Hewitt G. Fletcher, Jr., at the National Institute for Arthritis and Metabolic Diseases in Bethesda. Clinton E. Ballou came from Karl Paul Link's laboratory in Wisconsin. Link has the pleasant habit of passing on his very best co-workers to his friends. Ballou joined the Berkeley group in 1951 and is responsible for the fine papers in the inositol and hexose dialdehyde field, the successful synthesis of triose phosphates, and, together with MacDonald, the D-erythrose-4-phosphate work. Ballou, MacDonald, and Elvin A. Kabat are responsible for the galactinol paper. Dr. Ballou is now an Associate Professor of Biochemistry at the University of California in Berkeley.

The synthesis of D-erythrose-4-phosphate paid off marvelously in the biochemical sense. Srinivasan, Katagiri, and Sprinson, at Columbia University, condensed D-erythrose-4-phosphate with phospho-enolpyruvic acid (Baer and Fischer) under the influence of a cell-free extract of *E. coli* (mutant 83–24) to dehydroshikimic acid. Thus, a pathway from phosphorylated small carbohydrates to the hydroaromatic plant acids was established.

I was also fortunate in having as an honored guest and co-worker, Dr. Elvin A. Kabat of Columbia University. He, together with MacDonald and Ballou, participated in a paper on the structure of galactinol. This compound, interestingly enough, helped Dr. Kabat in his own work in immunochemistry. Another important visitor to the Department who stayed for half a year was Professor S. J. Angyal of the University of New South Wales in Sydney, Australia. He impressed all of our staff and students as an excellent lecturer. He modernized our course on carbohydrates and introduced his audience, in a masterful way, to the concept of Conformational Analysis of sugars and inositols. The research on sugars in Berkeley is deeply and favorably influenced by his teachings.

In 1956, time caught up with me and I had to retire, according to the University rules, at the age of 67. The chairmanship of the Department was taken up by Professor Esmond E. Snell of Texas, and originally from Wisconsin; this has proven to be an excellent choice. Considerable changes, in

particular in enlargement of the staff, have taken place. I have many favorable comments to make, but it is obviously beyond the scope of this autobiography.

Retirement does not mean inactivity nowadays since favorable circumstances, especially in California, have prolonged the life span of man. By gracious permission of the Chancellor, I was given a laboratory in the Biochemistry and Virus Laboratory, and, through the generous support of the National Institutes of Health, I was enabled to run a research laboratory on carbohydrates. Good fortune brought me an especially able co-worker in the person of Dr. Hans Helmut Baer. He came from Professor Richard Kuhn's laboratory in the Max-Planck-Institut for Medical Research in Heidelberg and managed in the first few months to enrich the interesting field of amino sugars by an entirely new and unexpected way of synthesizing 3-amino-3-deoxy sugars. Starting from my previous observation that a sugar dialdehyde of the pentose series forms, with nitromethane, nitro-inositols, Dr. Baer was able to condense, with nitromethane in aqueous methanolic solution, several of the well-known sugar dialdehydes prepared, according to Hudson and Jackson, from pentopyranosides and hexopyranosides by periodate oxidation. Indeed, in one of the first experiments, the sodium salt of the 3-nitro-3-deoxypentoside crystallized out. The ice was broken, and Dr. Baer could work out a new and practical synthesis of 3-amino-3-deoxy-D-ribose after reduction of the nitro group. In a similar way, he achieved the synthesis of 3-amino-3-deoxy-D-mannose from the dialdehyde produced by periodate cleavage of methyl-α-D-glucopyranoside. In view of the ease of the synthetic procedure and the importance of the 3-amino sugars as components of various natural compounds, this seems to be quite a satisfactory beginning. At the moment, various analogous sugar dialdehydes are being studied for the same purpose.

It is very important for a scientist to discuss his sometimes rather unfermented ideas with a friend. In the Toronto period, I traveled from time to time to New York to consult with my old friend, Max Bergmann, then at the Rockefeller Institute for Medical Research. He was not only a chemist with a fantastic knowledge of the literature but also a man with an awareness of the present and an insight into the future of science. It was extremely rewarding to discuss with him scientific and nonscientific problems. A treacherous disease took him away much too early.

In Berkeley, a similar warm friendship unites me with William Z. Hassid, with the additional pleasant factor that enzyme carbohydrate chemistry and organic carbohydrate chemistry complement each other very well.

Earlier in this little sketch, I tried to give the reader an impression of how full and rich in every respect were for me the years 1912 to 1914 in my father's laboratory in Berlin. At the time, I very likely did not understand their full significance. Here in Berkeley, the very clear understanding has come to me that this is the second most happy period of my life, endowed with all the stimulation offered by a great University, an active Institute, and a close association with my great friend Wendell M. Stanley.

MECHANISM OF ENZYME ACTION[1,2]

By Paul D. Boyer

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This is the first time this subject has been reviewed as an entity in these volumes. Such separation is a fitting recognition of the present interest and dynamic advance in this field. The scope of the chapter is broad, both because the useful and abused term "mechanism" has many levels of meaning,[3] and because aspects of mechanism are present in nearly every study of an enzyme system. Editorial fiat and the limitations of the reviewer, however, make the present coverage short and selective.[4]

This chapter is organized, not on the basis of a discussion of individual enzymes, but as a presentation of various approaches that may give insight into mechanism. Many studies of the mechanism of a given enzyme neglect valuable information from approaches not considered. Some of the approaches discussed are well-recognized; others are in the early developmental stage. Usefulness of the various approaches is illustrated by examples of significant advances with individual enzymes. Emphasis is on areas not recently or adequately reviewed elsewhere or where the author's experience may give him more competence for appraisal.

The evidence, both direct and indirect, that enzymic catalyses proceed through formation of enzyme-substrate combinations is overwhelming and will not be documented here. Our concern is how such combination favors catalysis. As pointed out in recent reviews [Lumry in (1); Koshland (2)], there is obviously much more to enzyme catalysis than the mere increase in concentration and positioning of reactants to enhance reaction by the same mechanism as in the absence of the enzyme. Broadly speaking, there are two means by which enzymic catalysis may occur: (*a*) by conditions that favor actual or potential change in electron densities at particular atoms of reactants, and (*b*) by steric displacements or constraints which effectively strain

[1] The survey of the literature pertaining to this review was concluded in December, 1959.

[2] The following abbreviations are used: AMP for adenosine monophosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); GSH for glutathione.

[3] The use and abuse continues in this review; thus, reference will be made to equations or statements as depicting mechanism of an enzymic reaction when it is clearly recognizable that many facets of the action of the particular enzyme remain unexplained. For a long time to come, explanation of enzymic mechanism will remain a a goal, not an accomplishment.

[4] Less than half of the more than 400 recent contributions considered for this chapter are included—authors and readers should be keenly aware that many pertinent papers are not cited.

bonds in reactants. These changes may be brought about in a wide variety of ways, and subtleties not yet visualized may be involved.

Delineation of the Reactions Catalyzed

Reactants, products, and their stoichiometry.—Documentation of all the different reactants that can be induced to undergo chemical change in the presence of a single enzyme or, better yet, single active site is of considerable pertinence to mechanism. Structural specificity studies remain one of the most promising ways for the "mapping" of enzyme sites. Occasionally, apparently unrelated reactions may be catalyzed. Among recent additions to the many examples that could be cited are the intriguing catalysis of the "fluorokinase" reaction by pyruvate kinase [Tietz & Ochoa (3)] and the demonstration that postulation of separate aldolases for cleavage of fructose-1-phosphate and 1,6-diphosphate is unwarranted [Peanasky & Lardy (4)]. Recent questions about the identity of the esteratic and peptide-bond splitting sites of proteases are undoubtedly discussed in the review on proteolytic enzymes.

Clear-cut knowledge of over-all reaction stoichiometry is essential to any mechanism study. Sometimes such information raises perplexing questions, as illustrated by the probability that mammalian carbamyl phosphate synthetase involves cleavage of two moles of ATP for each citrulline formed in contrast to use of a single mole of ATP by the bacterial enzyme (5). Similarly, an unusual, but unknown, mechanism must also apply for synthesis of S-adenosylmethionine, apparently by a single enzyme (6) that proceeds with a stoichiometry and isotope distribution as follows:

$$\text{Adenosyl-P}\text{—}\overset{*}{\text{P}}\text{—}\overset{*}{\text{P}} + \text{R—S—CH}_3 \rightarrow \text{Adenosyl-}\overset{+}{\text{S}}\begin{matrix}\diagup\,\text{R}\\ \diagdown\,\text{CH}_3\end{matrix} + \text{P—}\overset{*}{\text{P}} + \text{i}\overset{*}{\text{P}}$$

Deductions about intermediates.—A development of striking importance for the understanding of enzyme mechanisms is the demonstration that compounds which appear to be logical intermediate reactants and which, indeed, undergo appropriate change in the presence of an enzyme, may not be actual intermediates in the biochemically important reaction. Thus, the findings of Speyer & Dickman (7) with aconitase, as extended and confirmed by Englard & Colowick (8), give substance to suggestions of earlier workers that *cis*-aconitate is not an obligatory intermediate in the conversion of citrate to isocitrate. In such conversion, considerably less than one atom of deuterium from D_2O may be incorporated into each isocitrate formed—*cis*-aconitate should thus be removed from representations of the citric acid cycle. The interconversions of citrate, isocitrate, and *cis*-aconitate are logically visualized as involving a common carbonium ion intermediate (7).

The claim of Moyle & Dixon (9) that a single enzyme catalyzes both the oxidation of isocitrate to oxalsuccinate and the decarboxylation of oxalsuccinate is strongly supported by the studies of Siebert, Carsiotis & Plaut (10). Free oxalsuccinate is not an intermediate in the formation of α-ketoglutarate

from isocitrate, and an enzyme-bound oxalsuccinate is proposed as an intermediate. The closely related "malic enzyme" appears to follow a similar pattern, in accord with earlier suggestions of Ochoa and co-workers [see Rutter & Lardy (11)]. Also, in these enzymic reactions, a metal, Mn^{++} or Mg^{++}, is required for both the oxidative decarboxylation and dehydrogenation reactions. Rutter & Lardy (11) mention that the reaction can be visualized as an "almost simultaneous process with no detectable intermediate." This is both logical and attractive and, taken together with the demonstrated role of metals in the non-enzymic decarboxylation of α-keto dicarboxylic acids [see Westheimer in (1)] and the demonstration of direct hydrogen transfer in pyridine nucleotide reactions, suggests to the reviewer a mechanism for the enzymic reactions as depicted in the scheme for the "malic enzyme" reaction shown in Figure 1. Decarboxylation is facilitated by electron withdrawal

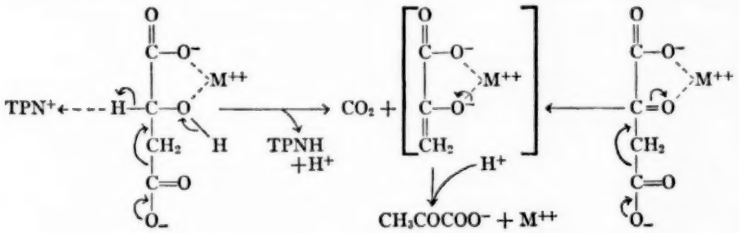

FIG. 1. A mechanism for the malic enzyme reaction.

from the β-carbon atom and concomitant withdrawal from the carboxyl group, which yields CO_2. Both transfer of a hydride ion to the pyridine nucleotide and chelation of the metal would promote electron deficiency on the α-carbon atom; thus, the dehydrogenation and decarboxylation processes can logically proceed simultaneously. The metal chelate of the enol form of pyruvate would be released to solution as the free pyruvate and metal.

An extremely important group of enzymes, whose recognized numbers are likely to increase considerably, are those which catalyze syntheses coupled to cleavage of nucleotide triphosphates, particularly ATP. These remarkable reactions, usually involving three substrates and three products, appear to be catalyzed by single enzymes. Nucleoside triphosphate cleavage patterns of these enzymes are discussed in a subsequent section; in this section, deductions about intermediates on the basis of reactions catalyzed are considered.

In one type of syntheses coupled to ATP cleavage, acyl adenylates have been accepted, perhaps too readily, as reaction intermediates. Important studies [see Berg (12, 13); Meister (14); Moldave *et al.* (15)] have shown enzymic conversion of acyl adenylates, in presence of pyrophosphate, to ATP or, in the presence of an acyl acceptor, to the expected product of the overall reaction. Formation of acyl adenylates from ATP and fatty acids has not

been achieved and from ATP and amino acids is achieved only with difficulty. In the closely related reaction catalyzed by glutamine synthetase, γ-glutamyl phosphate does not appear to be used or formed by the enzyme (14). "Tightly-bound" acyl adenylates or γ-glutamyl phosphate have thus been considered as intermediates. Evaluation of alternate possibilities led Boyer *et al.* (16) to suggest consideration of a sequence in which addition of ammonia to glutamate on the enzyme was necessary for ATP cleavage without formation of an enzyme-bound glutamyl phosphate as an intermediate. A related, but in some respects more attractive, suggestion for synthesis of glutamine and glycinamide ribotide has been made by Buchanan & Hartman (17): the reaction may be depicted as in Figure 2 for glutamine synthesis, where $RCOO^-$ is glutamate, and ADP—O—P is ATP, ADP—O is ADP, and $^-$O—P is inorganic phosphate:

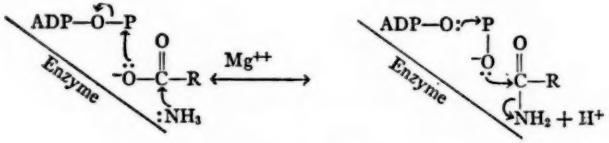

FIG. 2. Glutamine synthetase reaction.

Reaction sequences of this type may also be applicable to the thiokinases and amino acid activating enzymes. Thus, for acetate thiokinase, reaction might occur as depicted in Figure 3 where Ad—P—O—PP is ATP, Ad—P—O is AMP, and PP-O^- is pyrophosphate. Acetyl adenylate, as such, would

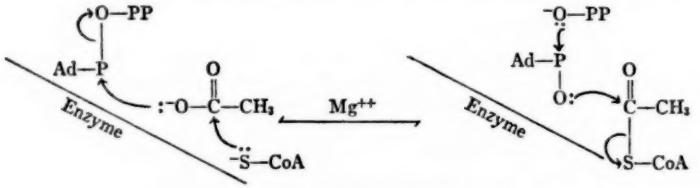

FIG. 3. Acetate thiokinase reaction.

clearly not be an intermediate in such a reaction. However, it could readily be expected to bind to the enzyme on the sites for adenylate and acetate and, when bound, to be readily cleaved by either pyrophosphate or CoASH. The above proposals also would account for the observed lack of formation of acyl adenylates [see (13)].

Evidence discussed by Berg (12) and other findings make it probable that the role of Mg^{++} in the reaction involves its association with the negative phosphate of ATP. Speculations on the role of Mg^{++} [Ingraham & Green (18)] have been rebutted by Berg (12). The mechanism proposed above, in

which acetyl AMP is not an intermediate, differs from that of both groups of workers, e.g.: "The formation of acetyl CoA from ATP, acetate, and CoA actually occurs in at least two discrete steps" (12); and "The intermediate is not acetyl AMP but the corresponding magnesium chelate, which has no measurable dissociation" (18).

Transfer reactions and intermediates.—The catalysis of transfer reactions by hydrolytic enzymes is frequently thought to demonstrate the formation of covalent intermediates of the groups transferred with the enzyme. As a recent example, Wallenfels & Malhotra, in continuation of their fine studies on β-galactosidase [see (19)], showed that those galactosides most readily hydrolyzed by a particular galactosidase were also formed most readily in transfer reactions. They suggest a galactosyl group is bound to an enzyme imidazole as an enzyme—SH donates an H to form ROH from the aglycone. The galactosyl group may then either be transferred to water or to the acceptor, which occupies the same site as the aglucone when the complete galactoside is bound. These attractive suggestions are in accord with experimental evidence, but the reviewer feels that, in such transfer reactions in general, consideration should be given to the possibility that the observations may reflect the relative rates of interchange of free and non-covalently bound reactants (see also the section on isotopic exchanges). With respect to galactosyl transfer by galactosidases, (G—O—R + R′—OH → G—O—R′ + R—OH), the non-covalent binding steps in the hydrolysis reaction may be visualized as indicated by steps 1, 2, 3, and 4 and the minimum covalent bond breaking steps by 5 in Figure 4. Transfer rather than hydrolysis would result if the

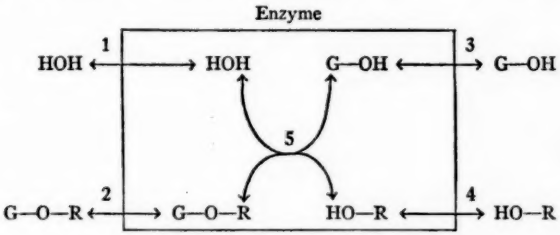

FIG. 4. Reaction steps in galactoside hydrolysis.

interchange in step 4 of R′OH with enzyme-bound ROH were rapid compared to interchange of free and bound GOH by step 3 and if appreciable reversal of steps 5 and 2 occurred. This would result in HOR′ appearing in unhydrolyzed G—O—R, i.e., galactosyl transfer. Postulation of a galactosyl enzyme is unnecessary, and the suggestion is in harmony with various findings in transfer reactions, including the need for high concentrations of acceptors for appreciable transfer. Interchange in step 4 might occur more readily than in step 3 if the energy barrier to the specific binding of the galactose in an activated form were considerably greater than that of the nonspecific

binding of the aglycone. Another alternate possibility to explain the transfer reactions is a compulsory binding order; if G—OH must be bound before R—OH can bind, presence of R—OH could inhibit the release of G—OH to the medium and favor exchange.

Transfer reactions catalyzed by sucrose phosphorylase (20) have been widely cited as occurring by intermediate formation of a glucosyl enzyme. Here again, logical alternate explanations have not received adequate consideration—sequences quite analogous to those suggested above for β-galactosidase could be applicable to a variety of other enzymes, including sucrose phosphorylase.[5]

Identification and Properties of Cofactors

Consideration of the mechanism of any enzymic reaction must take into account known or possible cofactors. Recent and comprehensive discussions of the properties and functions of enzyme cofactors are available (21), and only some highlights will be mentioned here.

One of the rewards of careful purification and study of enzyme composition is the delineation of the participation of cofactors. Study of the mechanism of any enzymic reaction is on shaky ground until the presence or lack of enzyme-bound cofactors is certain. The careful studies of Vallee and associates [see Vallee in (21)] exemplify rewards of careful analyses. A recent finding of marked potential importance is the correlation of Cu^{++} content of cytochrome oxidase with activity [Wainio *et al.* (22); Sands & Beinert (23)] and demonstration, by electron paramagnetic resonance, that the Cu undergoes cyclic oxidation and reduction (23). Such findings have obvious implications for the mechanism of enzymic reduction of oxygen to water. George [in (21)] gives a scholarly consideration of this problem and of electron transfer in enzyme catalysis in general.

With respect to the participation of free radicals in enzyme catalyses, recent work with flavoproteins [sees Beinert in (21)] gives convincing documentation for the participation of "semiquinone" forms of flavins in various flavin enzymes. In the enzymic reduction of DPN and TPN, hydride ion transfer without participation of a univalent reduction state appears likely [Westheimer in (1)]. Considerations of mechanism of the pyridine nucleotide-linked dehydrogenases should not be made without consultation of the recent extensive and valuable studies on model compounds by Wallenfels & co-workers [see (24)].

Past speculations on a role of biotin in the oxalacetate carboxylase reaction are rendered invalid by separation of enzyme activity from biotin through the ingenious use of avidin coupled to azobenzyl cellulose (columns) [Semenza *et al.* (25)]. Similarly, Hamilton & Westheimer (26) have isolated a

[5] Present data do perhaps favor the formation of a glucosyl enzyme in the sucrose phosphorylase reaction; the point to be emphasized is that reasonable alternative explanations must still be considered.

crystalline acetoacetate decarboxylase without biotin. Clarification of the role of biotin in CO_2 utilization promises to come, however, from the demonstration and study of an active biotin-CO_2 by Lynen *et al.* (27); by treatment with diazomethane, they isolated from their enzyme system a methylated biotin-CO_2 derivative.

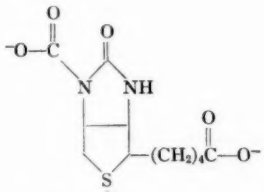

FIG. 5. Probable structure of biotin-CO_2.

The excellent and comparatively well-known studies on chemical structure and model systems of vitamin B_6 give basic information about the mechanism of the many and varied enzymes requiring pyridoxine or pyridoxal phosphates [see Braunstein in (21); Snell (28)]. Metal ions in conjunction with pyridoxal phosphate are particularly effective in model systems. Purification of enzymes requiring vitamin-B_6 derivatives has been unfortunately limited, but it is pertinent that the purified cystathionine and homoserine-cleaving enzyme, which contains four pyridoxal phosphates per mole of enzyme, does not contain or require any metal ion [Matsuo & Greenberg (29)]. The role of the bound pyridoxal phosphate of phosphorylase remains obscure [see Fischer *et al.* (30)].

The finding, by Breslow, of a rapid deuterium exchange at C-2 of thiazolium salts has allowed brilliant extension of studies on model systems for thiamine action [see Metzler in (21); Breslow (31)]. Thiazolium salts under mild conditions are in equilibrium with their dipolar ion forms, and the reactions of thiamine may be formulated in a manner analogous to those of cyanide ion for the classical benzoin condensation; thus, the decarboxylation of pyruvate to acetaldehyde or to acetoin may be visualized as depicted in Figure 6. Krampitz *et al.* (32) have shown that the hydroxyethyl thiamine derivatives, corresponding to addition of H^+ to the intermediate depicted in Figure 6, will activate yeast carboxylase.

Additional clues about the mechanism of other vitamin cofactors is accumulating. The information now available about the interconversion of various folic acid derivatives in relation to their metabolic function is gratifying compared to the confusion of a few years ago [see Rabinowitz in (21); Huennekens *et al.* (33)]. Much new information on a molecular level is still needed, and, indeed, the recognized type of action of folic acid derivatives may be broadened by the interesting findings of Kaufman (34) which demonstrate a tetrahydropteridine involvement in the direct hydroxylation of

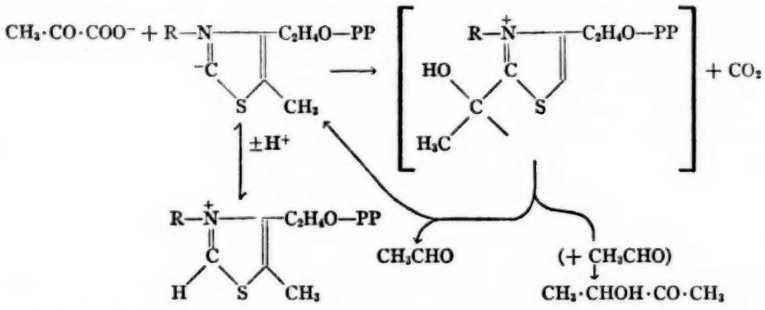

FIG. 6. Participation of thiamine pyrophosphate in pyruvate decarboxylation.

phenylalanine to tyrosine by O_2. A definitive clue to the action of vitamin B_{12} appears to be at hand from the observations of its relation to isomerization of β-methylaspartate and methyl malonyl CoA (35, 36). Recognition of the coenzymic role of GSH in the maleylacetoacetate isomerase and indolylpyruvic acid enol-keto-tautomerase [see Knox in (21)] suggests that the long-sought functions of GSH may lie in addition to carbonyl groups or double bonds, rather than in an oxidation-reduction role. A broadening of the understanding of lipoic acid function is likely to result from the observation by Massey and by Reed that diaphorase preparations have strong lipoic dehydrogenase activity; this and other aspects are ably discussed by Reed (21).

STRUCTURE AND PROPERTIES OF THE ENZYME PROTEIN

Although fine progress has been made in determination of the primary composition of enzymes, in particular ribonuclease, adequate description of the constellation of groups intimately involved in enzyme catalysis is yet to be achieved for any enzyme. Some important inferences about primary composition and active sites are discussed in the next section. The notable demonstrations that considerable portions of some enzymes can be removed without alteration of catalytic activity show clearly which residues do not have an essential role in the catalysis, but actually give little insight as to mechanism. An important point is, however, that the active degraded enzymes are still large in size and dependent upon secondary and tertiary structure for their activity. Also, requirements for the complete molecule may be less rigid for enzymes catalyzing a simple reaction, such as hydrolysis, rather than more complex reactions. Loss of activity accompanying degradation may be more significant to mechanism. Recent reports of interest are the removal of three COOH-terminal residues of aldolase with concomitant marked loss of activity and change of specificity [Drechsler *et al.* (37)] and

the reversible loss of ribonuclease activity upon removal of a 20-residue peptide fragment by subtilisin [Richards & Vithayathil (38)].

Another facet of protein structure-activity relationships, namely, the possibility of conformation change accompanying substrate binding, is of considerable pertinence to mechanism. Proteins are not to be regarded as fixed, rigid structures. Various regions of the protein will, in general, have varying degrees of configurational freedom, with little free-energy change involved in the transitions. This and other aspects of protein structure in relation to enzyme activity are admirably discussed by Lang & Schellman (1). Change in structure has been well-demonstrated for the binding of small molecules by serum albumin and configurational changes appear to accompany the binding of competitive inhibitors by chymotrypsin. However, the binding of the virtual substrate acetylbromotyrosine to chymotrypsin is not accompanied by an optical rotation change [see Lang & Schellman in (1)]. Independent studies of Boyer & Schulz (39) and of Elodi & Szabolcsi (40) show that the binding of DPN by glyceraldehyde-3-phosphate dehydrogenase is accompanied by a small increase in optical rotation; this may reflect a configurational change of the enzyme with binding. Interpretation of changes in optical rotation upon binding of substances that absorb light must be made with caution, as shown by the findings of Blout & Stryer (40a). With the dehydrogenases, the increased susceptibility of the protein to proteolytic attack in the absence of bound DPN also suggests protein structural change (41, 42). An unusual and significant effect of pyridine nucleotide binding on the association of glutamic dehydrogenase into the active enzyme has been described in an excellent study by Frieden (43); the nature of structural changes other than the size change is not known.

Configuration changes with binding could be of particular importance in explaining compulsory binding sequences or the lack of catalytic activity unless all substrates are bound, even though independent reaction steps involving only one substrate may occur with the active enzyme; the configuration change could bring the groups required for catalytic activity into proper orientation. Such ideas have recently been extended by Koshland, who gives an "induced-fit" theory as a possible explanation for some aspects of specificity [see Koshland in (1)]. Detection of small configurational changes accompanying binding of substrates and, even more pertinent, of the nature and mechanistic importance of the changes are tasks of considerable challenge and importance.

The extent of change in enzyme structure necessary for modification of enzyme activity could logically be very small. Results in harmony with this are the findings that concentrations of urea, which cause little change in optical rotation, reversibly inactivate aldolase [Swensen & Boyer (44)] and papain [Hill, Schwartz & Smith (45)]. The importance of proper positioning of groups from different chains is indicated by the studies of London *et al.* (46) with acid phosphatase; they regard the substrate as straddling a "critical-seam."

Smith (47) has suggested that thiol ester, acylimidazolyl, and acylphenolic bonds exist in enzymes as parts of active sites and that such covalent bonds are also involved in maintaining the three-dimensional structure of proteins. Evidence for the presence of a thiol ester or related linkage in papain is unconvincing, but the idea is stimulating. A built-in labile structure in the enzyme, the lability of which contributes to activation of substrate and which is regenerated in the catalysis, is intriguing but needs more experimental support to warrant serious consideration.

Questions of crystal-like electronic states and energy migration in enzymes as contributors to mechanism of enzyme catalysis have been ably considered by Lumry (1). Suffice it to say that even moderately convincing findings are not sufficiently available to warrant further discussion here.

Essential Sites and Groups

Present information favors the view that no one amino acid residue and its immediately adjacent residues of the same peptide chain can be regarded as an active enzyme site but that such sites involve groups that are separated by a considerable number of peptide bonds or are from different chains. These groups are brought into the necessary spatial proximity by secondary and tertiary structure to give the active site. Approaches at present are largely directed toward identification of important groups contributing to the active site of a given enzyme.

The pioneering demonstration by Jansen, Nutting & Balls (48) that chymotrypsin could be inactivated by attachment of one diisopropylphosphoryl residue per enzyme has, during the ensuing decade, led to identification of amino acid sequences containing active seryl residues of several enzymes. In these enzymes, the seryl residue appears to be the primary point of attachment of the diisopropylphosphoryl group. Present information is summarized in Table I. Particularly noteworthy in the five enzymes for which the sequence has been established is the occurrence of a dicarboxylic amino acid and an alanine or glycine adjacent to the serine. This is clearly not a sequence expected for all serine-containing peptides, as indicated by the absence of such a sequence in ribonuclease and insulin and surrounding the other seryl residues in the peptide from trypsin (Table I). However, such a sequence is probably not peculiar to enzyme active sites, as indicated by the presence of an adjacent dicarboxylic amino acid and alanine or glycine, as in phosphopeptides from pepsin and casein (Table I). The mode of participation of the seryl group in enzyme action has been most thoroughly studied with chymotrypsin (see pp. 45–72). Evidence indicates that a histidyl or other residue readily protonated near neutrality is brought into the vicinity of the seryl residue by the secondary and tertiary structure of the enzyme.

Similar sequences should not be anticipated in all enzymes catalyzing similar reactions. Thus, Jolles [in (19)] has noted that the sequence containing the unique histidine of dog spleen lysozyme is completely different from the corresponding sequence of hen egg-white lysozyme.

TABLE I

AMINO ACIDS NEAR REACTIVE SERYL RESIDUES OF PROTEINS

Protein	Amino Acids*
Trypsin (49)	$AspNH_2 \cdot Ser(DIP^{32}) \cdot Cys \cdot GluNH_2 \cdot Gly \cdot Gly \cdot Asp \cdot Ser \cdot Gly \cdot Pro \cdot Val \cdot Cys \cdot Ser \cdot Gly \cdot Lys$
Chymotrypsin (50)	$Gly \cdot Asp \cdot Ser(DIP^{32}) \cdot Gly \cdot Gly \cdot Pro \cdot Leu$
Liver Aliesterase (51)	$Gly \cdot Glu \cdot Ser(DIP^{32}) \cdot Ala \cdot Gly \cdot Gly \cdot (Glu, Ser)$
Pseudocholinesterase (52)	$Phe \cdot Gly \cdot Glu \cdot Ser(DIP^{32}) \cdot Ala \cdot Gly \cdot (Ala_2, Ser)$
Thrombin (53)	$Gly \cdot Asp \cdot Ser(DIP^{32}) \cdot Gly \cdot (Glu_x, Ala_x)$†
Elastase (54)	$Gly \cdot Asp \cdot Ser(DIP^{32}) \cdot Gly$
Bacterial Proteinase (55)	$DIP^{32} \cdot Ser \cdot (GluNH_{2x}, Gly_x, \text{and others})$†
Phosphoglucomutase (56)	$P^{32}\ Ser \begin{cases} (Asp_x, Ser_x, Gly_x) \\ (Glu_x, Ala_x, Val_x) \end{cases}$†
Phosphorylase-*a* (57)	$Lys \cdot GluNH_2 \cdot Ileu \cdot Ser(P^{32}) \cdot Val \cdot Arg$
Pepsin (58)	$Glu \cdot Ser(P) \cdot Thr$
Casein Phosphopeptones (59)	$P^{32} \cdot Ser \begin{cases} (Gly_x, Ala_x, Val_x) \\ (Asp_x, Glu_x) \end{cases}$†

* DIP is used for the di-isopropyl phosphoryl group.
† Number and sequence of amino acids not determined.

Speculation that histidine residues are involved in action of a number of enzymes has become a favorite pastime, principally because various model systems show that such residues can function as catalysts and because pH-activity relationships indicate participation of a group with a pK value in

the range of 6 to 7 in the catalysis. Enthusiasm for such an interpretation of pH data has shown what appears to be an unjustified increase lately, even though more than one good reason suggests additional caution is in order. It has long been recognized that charged groups in proteins may show anomalous pK values [see (60)]. For example, —COOH groups in pepsin and in paramyosin have apparent pK values in the range 6 to 7 [see (61, 62)]. Tanford (63) has given an excellent theoretical presentation of how electrostatic factors may account for the apparently anomalous dissociation constants. Hydrogen bonding with other groups may also be involved [Laskowski & Scheraga (64)]. In addition, Klotz (65) has interpreted such effects as resulting from the properties of water bound to the proteins. Another important consideration is brought into focus by Bruice & Schmir (66), who have pointed out that the particular mechanism involved may change the apparent pK of the participating group in an enzymic reaction. In addition to these considerations, the possibility that the change in activity results from change in conformation secondary to change in charge on a group some distance from the active site, is usually ignored or mentioned briefly and then quickly forgotten. All this does not mean that pH-activity relationships are useless but that additional data must be sought to allow more positive identification of the participating groups. One such approach is that used in recent studies on papain in which the heat of ionization of the groups is approximated [Smith, Chavre & Parker (67)]. However, heats of ionization of protein groups may differ from those of corresponding groups in simple peptides (60).

Additional means of evaluating a role for histidyl groups may also be sought. For example, a role in ribonuclease is indicated by the demonstration that alkylation of one of four histidyl residues gives an inactive protein (68). Photo-oxidation may also be used, as with chymotrypsin (69); such oxidation also attacks tryptophan residues, however. The latter are more specifically oxidized in chymotrypsin by peroxidase (70). Results of photo-oxidation and related studies support a role for histidyl residues in DPNases (70a).

Recent reviews give a critical appraisal of the relations of the sulfhydryl group [Boyer in (1)] and of other reactive groups [Fraenkel-Conrat in (1)] to enzyme catalysis. The versatile and reactive —SH groups are probably involved in a number of enzyme catalyses, but the paucity of convincing evidence shows the difficult nature of the task of assigning a definite function to a particular group. Perhaps the best established role for an —SH group is as an acyl acceptor in glyceraldehyde-3-phosphate dehydrogenase [see Boyer in (1); Racker and co-workers (42)]. Researches of Smith's group (71) point strongly to a role of —SH in catalysis by papain, but the suggestion that " . . . it is more likely that the active site of papain (and of other so-called 'thiol enzymes') involves a preformed thiol ester rather than a thiol group per se . . . " may be overenthusiastic. Among other things, postulation of thiol ester is unnecessary to account for the reactivity of an —SH

group of papain toward mercurials and other —SH group reagents; the thiol of papain has not been demonstrated to be more reactive than —SH groups of small molecules or peptides.

Other meritorious recent contributions include data that indicate a role of —SH in substrate binding by microsomal cytochrome reductase [Strittmatter (72)] and lactate dehydrogenase [Winer & Schwert (73)]. Fridovich & Handler (74) give a stimulating proposal for the involvement of —SH, Fe, and flavin-adenine-dinucleotide in the active site of xanthine oxidase, and Hellerman *et al.* (75) speculate on —SH function in enzyme catalysis by nucleophilic displacement.

Source of Atoms in Products

Knowledge of the origin of all the atoms in a product is vital to the understanding of any enzyme reaction, and, until such information is available, either on an experimental basis or from highly probable prediction, any postulated mechanism is questionable. Unfortunately, the isotopic studies required are frequently not simple, particularly with the stable isotopes; fortunately, the information obtained is definitive and lasting.

Recent examples add to the well recognized fact that source of carbons is particularly basic to mechanism. Experiments of Beck & Ochoa (76) indicated that isomerization of methylmalonyl CoA to succinyl CoA might involve a transcarboxylation, but more recent findings (189) show that the reaction involves shift of the —CO—S—CoA moiety from the methine to the methyl group of malonyl CoA to give succinyl CoA. Ingenious studies of the enzymic cyclization of squalene to lanosterol by Maudgal, Tchen & Bloch (190) and by Cornforth *et al.* (77) using C^{13} allowed them to deduce whether the reaction occurred by two 1,2-methyl shifts or by a single 1,3-methyl shift. Degradation of the lanosterol to two-carbon fragments and mass spectrometric measurement of the amount of fragment doubly labeled with C^{13} allowed the conclusion that a double 1:2-methyl shift had occurred. In a related study on squalene biogenesis from mevalonic acid, determination of ratios of C^{14} and deuterium in reactants and products allowed, among other things, conclusions that head to tail condensations of isoprenoid units take place without loss of hydrogen bound to C-5 of mevalonic acid [Rilling & Bloch (78)].

The excellent studies of Topper and Bloom and of Rieder & Rose (79, 80, 81) on enzymic isomerization of carbohydrates further demonstrate the value of determining the source of hydrogen atoms in products. Thus, in the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by triose phosphate isomerase, one hydrogen from the medium is incorporated stereospecifically into the $-CH_2OH$ group of the dihydroxyacetone phosphate. This and other findings point strongly to the removal and addition of a proton rather than a hydride ion shift as the reaction mechanism. The enzymic isomerization of the hexose phosphates proceeds in an entirely

analogous fashion. Whether the reaction occurs with formation of a resonant enediolanion structure or by a concerted removal and addition of protons to the two carbon atoms is uncertain, but exchange and reaction rate data favor the former (81). To explain the formation of a particular epimer by a given isomerase, Topper (79) favors participation of either a *cis*- or a *trans*-enediol intermediate, depending upon which epimer is formed. Nothing is known about the groups on the enzyme responsible for proton withdrawal. Speck (82), in a review on such isomerizations and epimerizations, objects to this interpretation, principally on the basis that a ketose cannot be an intermediate in the alkali epimerization of such compounds as 2,3,4,6-tetra-O-methyl-D-glucose. To the reviewer however, it appears plausible that different intermediate structures may be involved in the alkali and in the enzyme catalyzed reaction.

Other recent studies on enzymic reactions show that condensing enzyme does not cause an enolization of acetyl coenzyme A in the absence of oxalacetate (83, 84) and that the keto form of oxalacetate is the substrate (85). In the glyoxylase reaction, the results of Franzen and of Rose, showing that the α-H of lactate formed does not come from H_2O of the medium, prove that a free enediol is not an intermediate, and have led Knox to propose that the reaction involves a hydride shift in a manner analogous to the well-known benzilic acid rearrangement, where GS^- plays the role usually assigned to ethoxide ion, shown in Figure 7 [see Knox in (21)].

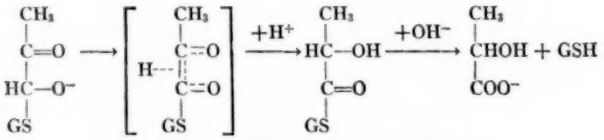

FIG. 7. Proposal for glyoxalase reaction.

A variety of studies have demonstrated the usefulness of the O^{18} isotope for determination of position of bond cleavage and source of oxygen in products. In syntheses coupled to cleavage of nucleoside triphosphates to nucleoside monophosphates and pyrophosphate or to nucleoside diphosphates and inorganic phosphate, the generalization may be made that oxygen from one of the substrates is incorporated into a phosphate group. Further, only two of four probable patterns of oxygen transfer have been observed. When pyrophosphate is formed, oxygen from substrate interchanges with the phosphate of the nucleoside monophosphate and not the pyrophosphate in the following reactions: acetate$\leftrightarrow$acetyl CoA (86); amino acids$\leftrightarrow$acyl hydroxamates (87, 88); β-alanine$\leftrightarrow$pantoic acid (89); and xanthosine 5′-phosphate$\leftrightarrow$adenosine 5′-phosphate (90). When inorganic phosphate is formed, oxygen from substrate interchanges with inorganic phosphate and not the nucleoside

diphosphate in the following reactions: glutamate↔glutamine (86, 91); succinate↔succinyl-CoA (92, 93), γ-glutamylcysteine↔glutathione (94); glutamic acid↔γ-glutamylcysteine (94); inosine 5′-phosphate↔adenylosuccinate (95); and glycine↔glycinamide ribonucleotide (96). The data are consistent with formation of phosphoryl or adenyl intermediates, but, as pointed out earlier (see p. 18), the syntheses may involve a more or less simultaneous series of displacements.

The very pertinent demonstrations of direct hydroxylation by molecular oxygen demonstrate how the source of atoms is basic to understanding mechanism [see (97)]. Other recent uses of O^{18} include the surprising finding that urease will catalyze transfer of oxygen from inorganic phosphate or arsenate to the CO_2 formed (98) and that, in the cleavage of ATP by actomyosin-Mg^{++} (99) or of casein phosphate by phosphatase [see (100)], more than one oxygen from water appears in each inorganic phosphate formed. In the enzymic formation of acetoacetate and succinyl CoA from acetoacetyl CoA and succinate, oxygen in the acetoacetate carboxyl group is derived from succinate, and a mixed acetoacetic-succinic anhydride is suggested as an intermediate [Falcone & Boyer (101)].

In oxidative phosphorylation catalyzed by liver mitochondria [Boyer (102)] or in the formation of ATP by intact frog muscle [Fleckenstein *et al.* (103)] as well as in the light-induced formation of ATP by chloroplasts [Schulz & Boyer (104)], the oxygen between the two terminal phosphate residues of ATP is furnished by the ADP. This gives the most definitive proof yet available that these important processes involve chiefly, or solely, activation of, or formation of, a covalent intermediate by inorganic phosphate. None of the evidence to date eliminates, however, the possibility that the first covalent bond formed in these processes is that between inorganic phosphate and ADP (102).

Stereochemistry of Reactants and Products

The stereospecificity of enzyme action, although long recognized, was brought into sharp focus by Ogston's brilliant deductive reinsertion of citrate into the citric acid cycle. Subsequently, continued experimental probing has given superb documentation and clarification of specific instances of stereospecificity. Recent additions to knowledge of enzyme stereospecificities are demonstrations of the absolute configurations of isocitrate participating in the aconitase reaction [Gawron *et al.* (105)] and of dihydroxyacetone phosphate tritiated by the aldolase reaction [Rose (106)]. Of interest also is the finding that the addition of water in the fumarase reaction is "*trans*" (107); earlier studies had indicated a "*cis*" addition (108).

Perhaps the most cogent relation of stereospecificity to mechanism is the convincing evidence that this can result only from the proper three-dimensional orientation of interacting groups at the binding site. Implications about the direction of approach and departure of reactants are also evident

but, unfortunately, not as explicit as often assumed. Koshland [in (1); (109)], several years ago, called attention to the possibility that inversion or retention of configuration in the enzymic hydrolysis of glucosyl-O bonds could logically be related to occurrence of the reaction by a single or double displacement, respectively. However, the question definitely remains open of whether retention of configuration, as in the phosphorylytic cleavage of the 1,4 linkage of glycogen to give α-glucose-1-phosphate, results from a double-displacement with participation of a glycosyl-enzyme intermediate or by a "front-side" attack, possibly involving a transient carbonium ion. Mayer & Larner (110) favor participation of a carbonium ion followed by solvolysis for the phosphorylase as well as the α- and β-amylase reactions.

Combinations with Substrates, Cofactors, and Inhibitors

The demonstration of enzyme combinations, both covalent and noncovalent, is of obvious importance to mechanism, in particular when combined with studies of kinetics and of properties of the combinations. Methods used for direct demonstration of such combinations include: (*a*) isolation and analysis of the combined form; (*b*) equilibrium measurement of binding by equilibrium dialysis or ultracentrifugation; (*c*) light absorption change accompanying binding; and (*d*) changes in fluorescence emission spectra accompanying binding. Indirect evidence for formation of such combinations come from: (*a*) change in properties of the enzyme protein in presence of substrates and cofactors; (*b*) reaction stoichiometry with high levels of enzymes; (*c*) various kinetic studies; and (*d*) demonstration of exchange reactions. Some recent researches illustrative of the value and limitations of the various approaches are given in this section.

The important demonstration of Jagannathan & Luck (111) that P^{32} of glucose-1-phosphate-P^{32} became incorporated into phosphoglucomutase during catalysis was a forerunner to the present extensive information documenting the participation with both the yeast and muscle enzyme of a phospho-enzyme as an intermediate [see Najjar & McCoy (112, 113)]. Studies with this enzyme give the best evidence yet attained for the participation of a covalent enzyme-phosphate in any catalysis, even though it is not known whether the enzyme-phosphate would meet the requisite kinetic test of an intermediate, i.e., that it appears and disappears at rates consistent with the over-all catalysis rate. Phosphoglyceric mutase appears to have a mechanism analogous to phosphoglucomutase [Pizer (114)]. Evidence presented for participation of phosphoenzymes in the action of kinases is, however, unconvincing. Agren's (115) claim for the formation of a phosphohexokinase, based on isolation of serine-P^{32} from treatment of an impure preparation with ATP^{32}, does not appear valid [Hass & Boyer (116)]. A similar report for phosphorylase [Engström & Agren (117)] has also been shown not to hold for well-purified phosphorylase [Krebs *et al.* (118)]. These findings emphasize the importance of use of well-purified enzymes for such studies. The findings of Agren *et al.* (119) that phosphate is incorporated into

serine in phosphatase preparations may not be taken as conclusive evidence for participation of a phosphoenzyme intermediate, although the rapidity and extent of labeling is suggestive.

An interesting suggestion that the action of glutamine synthetase involves an enzyme-S—PO_3H^-, which is converted to an enzyme-S—P—O—CO—R, has been made by Wieland *et al.* (120). Experimental evidence is chiefly of differential migrations on starch column electrophoresis, such as migration of P^{32} with the enzyme and separate from ATP after mixing with ATP^{32}. The possibility remains, however, that migrations with the enzyme reflect non-covalent specific or non-specific binding of inorganic phosphate-P^{32} or ATP^{32} rather than formation of a covalent enzyme-phosphate. Other important isolations of enzymes bound covalently to a moiety from the substrate or inhibitor include the isolation of the diisopropylphosphoryl enzymes mentioned earlier and the isolation of acylated glyceraldehyde-3-phosphate dehydrogenase [see (70)] and chymotrypsin [see (121)], which appear to be catalytic intermediates. These enzymes also serve as outstanding examples of the use of observable reaction stoichiometry with high levels of enzymes as evidence for acyl enzyme formation.

Isolation of combined forms with unchanged substrate may, in some instance, be possible, as shown by the remarkable isolation of three different acyl dehydrogenases with firmly bound, but apparently uncleaved, acyl-CoA substrates in addition to the bound flavin [Steyn-Parvé & Beinert (122)]. Of interest is the finding that, contrary to earlier reports, copper is not a constituent of the green flavoprotein, butyryl dehydrogenase; the nature of the material responsible for the green color is unknown (123).

Recent examples of valuable quantitative studies using equilibrium dialysis and ultracentrifugation techniques include clarification of the binding of DPNH to yeast glyceraldehyde-3-phosphate dehydrogenase [Stockell (124)] and substrate binding by creatine kinase and adenylate kinase [Kuby & Mahowald (125)].

Change in light absorption, which sometimes accompanies binding of a substrate to an enzyme, gives a powerful tool for demonstration of formation of combinations and for study of their properties. Usefulness of such approaches for studies with pyridine nucleotide linked dehydrogenase is exemplified by well-known studies with alcohol dehydrogenase [see (126)]. The binding of any substrate to an enzyme will probably result in shifts in energy levels of bonding electrons; thus, with any substrate, which absorbs readily measurable ultraviolet or visible light or for which light-absorbing intermediates might be expected, experiments to detect such possibilities would appear to be in order and technically feasible in many laboratories. Another example of the value of such experiments, with careful consideration of artifacts, is the demonstration by Topper, Mehler & Bloom (127) of an absorption by aldolase plus dihydroxyacetone phosphate indicative of formation of a bound carbanion or enediolanion.

Decrease of fluorescence associated with the binding of substances, such

as riboflavin and DPNH has been extensively used for binding studies. More recent recognition that the binding of DPNH by alcohol dehydrogenase is accompanied by a change in fluorescence emission spectra [Boyer & Theorell (128)] has led to a series of excellent studies based on such shifts and related phenomena, particularly in the laboratories of Schwert (129), Velick (130), and Theorell (131, 132). Also malic dehydrogenase from heart is to be added to the list of enzymes showing such shifts (133). Winer & Theorell (132) report that fatty acids combine with the ethanol binding site of alcohol dehydrogenase-DPN but that the corresponding amides combine only with alcohol dehydrogenase-DPNH. The latter complexes are regarded as similar in structure to the activated complex involved in the conversion of the dehydrogenase-DPNH-aldehyde to dehydrogenase-DPN-alcohol, analogous to the fluorescent lactic dehydrogenase-DPNH-anion complexes described by Winer & Schwert (129). Expansion of fluorescence techniques shows considerable promise, but, unfortunately, readily available instrumentation for careful fluorescence activation and emission studies is inadequate.

Kosower [in (21)] has suggested that complexes of pyridine nucleotides with enzymes are, in part, charge transfer complexes and he interprets mechanism of action of these dehydrogenases on this basis. This concept has been criticized by Kaplan [in (21)].

Any methods that will reveal the properties of enzyme-substrate or enzyme-cofactor combinations, either in the static state or during catalysis, are of obvious importance. Deductions can sometimes be made from light absorption and emission studies. Measurement of electron spin resonance will undoubtedly be increasingly applied to detect valence changes in enzymic reactions. The demonstration of valence change of copper in cytochrome oxidase preparations was mentioned earlier (23). Other recent examples are the demonstration of valence change of Cu in laccase action and the occurrence of a flavin and, possibly, of a Mo valence change in reduced xanthine oxidase by Malmstrom and associates (134, 135). Results of Commoner *et al.* (136, 137), indicating presence of free radicals, probably enzyme-bound, in a number of systems, have been explained by Miyagawa *et al.* (138) as the result of oxygen trapped in the system, a point disputed by Commoner *et al.* (137). Data of Commoner's group do give strong support for the presence of free radicals during catalysis by several oxidative enzymes. The relation of the radicals to the catalysis is not clear.

That water may participate in enzymic hydrolyses through a specific interaction with the enzyme has long been regarded as probable. Koshland & Herr (139) describe an ingenious approach to this problem based on the ability of myosin-adenosine triphosphatase to catalyze transfer of phosphate to methanol as well as to water. With the use of radioactive methanol, the relative reactivity of water to methanol was of the order of 300 to 1000 for the enzymic reaction, but only 0.4 to 2.5 for nonenzymic cleavage of phosphate compounds. This shows a specific myosin-water interaction.

Occurrence and Rate of Isotopic Exchanges

Exchange of groups of substrates.—One of the results of the introduction of isotopic tracers as common tools for biochemical studies has been the discovery that some enzymes will catalyze the exchange of a moiety of one substrate with its free counterpart in the absence of a second substrate or substrates. Such exchanges provide valuable clues about enzyme mechanisms, but their occurrence has been too readily accepted as proof for formation of covalently bonded intermediates either with the enzyme or with another substrate. For example, following the views of various researchers, Dixon & Webb (140) state that such exchange methods allow decision between reactions in which there is a direct transfer of the group from one molecule to the other and reactions in which an intermediate compound of the group with the enzyme is formed. Perhaps a more appropriate view is that occurrence of substrate-independent exchange reactions is a requirement of, but not proof for, the formation of covalent intermediates of the exchangeable moiety independent of the presence of the missing reactant.

Alternate explanations for exchange reactions other than postulation of covalent intermediates have received far too little attention. As discussed on p. 19, the transfer reactions of β-galactosidase could result from differences in the rates of interchange of noncovalently bound substrates with free substrates. A similar explanation could be advanced for the exchange reactions of sucrose phosphorylase (20). Further, another alternative is that the exchangeable species itself directly attacks the bond cleaved by a "front-side" displacement or reacts with a transient carbonium ion in an ionic S_N1 mechanism as suggested for amylase and phosphorylase action (110, 141).[5]

An example of another type of exchange reaction, namely, that occurring in a hydrolytic reaction, is the incorporation of glucose into glucose-6-phosphate by glucose-6-phosphatase more rapidly than explainable by the over-all reverse reaction [Hass & Byrne (142)]. Attractive explanations of this exchange and the lack of competition between glucose and glucose-6-phosphate have been given by Hass & Byrne (142) and by Segal (143), who based their findings on formation of a phosphoryl-enzyme. As an alternate to phosphoryl-enzyme formation, the possibility exists that the observations may result from a relatively slow interchange of phosphate with non-covalently bound enzyme phosphate; this could occur in a manner quite analogous to that suggested for transfer reactions by β-galactosidase (see p. 19).

Exchanges suggestive of formation of phosphoenzymes have not been found with kinases (112, 144, 145). Formation of phosphoenzyme intermediates is possible, however, if a compulsory order of addition of substrates occurs or if a requisite change in enzyme configuration accompanies binding of adenine nucleotides. Participation of a phosphoenzyme intermediate in intramolecular transfer, as in the phosphoglucomutase reaction, but not in intermolecular transfer of phosphate may reflect the limitations imposed by bringing the recipient —OH group into position for nucleophilic displace-

ment on the P atom of the same molecule, together with proper orientation of groups required for the activation and specific binding of the substrate.

The exchange of pyrophosphate with ATP, as found with thiokinases and amino acid activating enzymes (5 to 8), is consonant with the formation of an acyl adenylate, but, as discussed on p. 17, an ability to form or to use an acyl adenylate does not show that it is an intermediate in the reaction.

Exchange reactions associated with oxidative phosphorylation have given limited useful information in this difficult area [see (102)]. The assumption by Wadkins & Lehninger (146), that an ADP-ATP exchange observable with preparations from mitochondria definitely represents a portion of the oxidative phosphorylation process, needs to be accepted with reservation until more evidence is available.

Exchange of atoms of substrates.—Evaluations of possible exchanges of atoms of one substrate in presence of enzymes, but in absence of other substrates, is becoming more common. Such evaluations are to be commended as giving insight into mechanism. To cite one example, in an excellent extension of earlier observations that aldolase catalyzes a stereospecific exchange of a hydrogen of dihydroxyacetone phosphate, Rose & Rieder (147) studied the rates of the exchange under various conditions. The results enhance the possibility that the observed exchange reaction is indicative of the mechanism and, in addition, demonstrate that the exchange rate at different substrate concentrations leads to the enzyme-substrate dissociation constant. Vaslow (148) showed this relationship earlier with exchange of oxygen in the carboxyl group of N-acetyl-3,5-dibromo-L-tyrosine by chymotrypsin.

Absence of exchange can also be of mechanistic significance in the elimination of mechanisms. Thus, Alberty *et al.* (149) found it necessary to introduce a further intermediate in their proposed mechanism for fumarase because of lack of an H-exchange and a deuterium rate effect. To evaluate the possible occurrence of H-exchange, Alberty & Miller (150) developed relationships governing the first order interconversion of three substances in the presence of an over-all reaction.

Exchanges of products and reactants.—Measurement of the rate of incorporation of an isotopically labeled product into a reactant or vice versa is a well-recognized procedure for evaluation of reaction rates in systems where the reaction is preceding in both directions. Relatively little use has been made of this approach in association with enzyme studies, but recent developments suggest that considerable important information may be attained by such studies. Hager (93), in 1957, showed that the rate of exchange of phosphate oxygen with succinate exceeded the rate of exchange of inorganic phosphate with ATP in the presence of succinate thiokinase. More recently, Rose (151) demonstrated unequal interchange between fructose-1,6-diphosphate and the two triose phosphates formed by the cleavage reaction of aldolase. Boyer *et al.* (16) demonstrated that, in the glutamine synthetase reaction at equilibrium, the rate of interchange of inorganic phosphate with ATP could be less than or greater than the rate of interchange of glutamate and glutamine.

These studies suggest, among other things, that steps of association and dissociation of substrates from enzymes, rather than covalent bond-forming steps, govern the rate of the exchange reactions.

Theoretical aspects of the uses and limitations of rates of isotopic exchange and incorporation in catalyzed reactions are discussed by Boyer (152). Important deductions relevant to enzyme mechanism are that exchange rates at equilibrium can lead directly to enzyme-substrate dissociation constants and that measurement of such rates may reveal compulsory orders of addition of substrates to enzymes. Systems at equilibrium have distinct advantage over the usual study of rate of product formation because in the former all reactants and intermediates are at equilibrium and not steady-state concentrations.

Kinetics of Intermediate and Product Formation

Kinetics, often viewed with awe, has power but limitations, which are often little recognized. Enzyme mechanisms are not proved by kinetics alone, but any mechanism must meet kinetic tests. Abuses arise from poor primary data and experimental design, unwarranted or unrecognized assumptions, and insufficient consideration of alternate explanations. The quality of enzyme kinetic studies appears to be increasing, although not infrequently the actual or potential yield from the mental gymnastics is low. The quantity of studies is rapidly increasing, and this section is perhaps the most incomplete of the review.

Segal [in (1)] has recently presented a readable essay on the historical development of enzyme kinetics, and Hearon *et al.* [in (1)] have given a comprehensive generalized analysis and extension of kinetics for enzyme systems, which should prove useful for many years. A recent book by Reiner (153) gives a competent and somewhat different view, and Dixon & Webb's (140) fine text contains considerable kinetic discussion. Laidler's (154) 1958 text on enzyme kinetics gives a competent discussion of the field. Chance's group has made commendable progress in kinetic study of metabolic control systems including a complete representation of the kinetics of 26 chemical components in 17 equations [see (155)].

Over the past several years, Alberty has contributed a series of experimental and theoretical studies on enzyme kinetics that should be consulted by any serious student of the field [see Alberty in (1); (156, 157)]. The most recent contribution of his group [Peller & Alberty (157)] gives a derivation for the general steady-state rate equation for reversible enzymic reactions with an arbitrary number of intermediates. The observable kinetic parameters, K_m and V_{max}, define lower limits to the rate constants for the unimolecular and bimolecular steps. The magnitude of the latter for a number of enzyme systems, including fumarase, suggests that a diffusion-controlled combination of enzyme and substrate is being observed. Frieden (158) has shown that, provided enough data are available and reasonable assumptions are valid, one can discern whether one or both ionic forms of a substrate are

utilizable. Krupka & Laidler (159) have developed equations for strong competitive inhibitors present in concentrations comparable to those of the enzyme. Pertinent developments of equations for evaluation of reaction parameters for a variety of substrates with an enzyme are given in the various papers by Niemann and co-workers [see (160)].

A principal kinetic goal is to determine the rate constants for all discernible steps in forward and reverse reactions. This goal has been approached for alcohol dehydrogenase by Theorell and associates [see (131)] and for fumarase by Alberty's group [see (156)]. A recent evaluation of the dissociation constants of liver alcohol dehydrogenase based on shifts in fluorescence of DPNH upon binding gives evidence, previously lacking, of the participation of the ternary dehydrogenase-DPN-ethanol complex in the reaction [Winer & Theorell (132)]. Other enzymes for which a considerable body of kinetic information is available include acetylcholinesterase [see Wilson (161)], lactic dehydrogenase [see Winer & Schwert (129)], and chymotrypsin [see Spencer & Sturtevant (121)].

The excellent series of experimental studies and associated theoretical treatments of Schwert and co-workers [see (129, 162)] with lactic dehydrogenase have established, among other things, that combination with DPNH is necessary before pyruvate will bind. Theoretical considerations in connection with a study on oxamate and oxalate inhibition show that the action of an inhibitor, which competes with a second reactant in a compulsory reaction pathway, cannot be characterized by the same constant for the forward and reverse reactions (162). Extensions of earlier work of Schwert's group on heart lactic dehydrogenase indicates that a compulsory binding order exists for erythrocyte lactic dehydrogenase [Ottolenghi & Denstedt (163)]. Kinetic studies in some systems may not allow definitive choice between compulsory and random order of addition, as illustrated by the recent careful kinetic studies of ribitol dehydrogenase by Nordlie & Fromm (164). Frieden's (165) ingenious experimental and kinetic treatment of the glutamic dehydrogenase system favors a compulsory binding order. The concept of a compulsory binding order harmonizes well with mechanism in which the first substrate forms part of the binding site for the second—such catalyses as intermolecular group or H transfer readily fit such a concept. Also, as briefly mentioned earlier, combination of one substrate may cause key conformation change in the protein.

Methods of elucidation of the role of metals by kinetic approaches are well illustrated by the careful kinetic studies of Noda (166) with adenylate kinase, which are compatible with participation of one nucleotide as a Mg complex and the other as a free nucleotide. Kinetic data of Hoch *et al.* (167) support the concept that metal is part of the active site of alcohol dehydrogenase, and the kinetic studies of Davis (168) considerably extend our information about carbonic anhydrase.

Continued applicability of rapid spectrophotometric techniques, elegantly exploited in well-known studies of Chance, is illustrated by the recent

study of Gutfreund & Sturtevant (169) on xanthine oxidase. Many of the earlier troubles in interpretations with this enzyme are explicable by the presence of slowly reduced flavin adenine dinucleotide on inactive enzyme. Presence of inactive or partially active enzymes with bound reactants could complicate many kinetic studies.

Of the many papers on mechanism of the chymotrypsin hydrolysis of *p*-nitrophenol esters (see pp. 45–72) this reviewer is inclined to favor the interpretation given by Spencer & Sturtevant (121) from their stop-flow kinetic studies; an acyl-serine is considered the only covalent intermediate, and its cleavage is rate-determining. Consideration needs to be given, however, to the possibility that the much more rapid hydrolysis of the peptide substrates may proceed without actual covalent ester formation. In this regard, mention may be made of the studies of Craig & Kistiakowsky (170) with liver esterase, which show that if an acyl-enzyme intermediate is formed, its rate of decomposition cannot be the rate-determining step.

Perhaps this review is a pertinent place to make a plea that the time-honored practice of equating Michaelis constants with dissociation constants for enzyme-substrate be discontinued. Some commit this "sin" even though they are aware that the Michaelis constant may be larger than the dissociation constant. Fewer appear aware, as clear from Haldane's fine 1930 treatise (171), that, in systems with one or more intermediate between "ES" and "EP," the Michaelis constant may be smaller than the dissociation constant. Other possibilities giving this disparity are noted in more recent papers (158, 172). Further, as pointed out by Hearon *et al.* [in (1)] and by Peller & Alberty (157), for interconversion of one product and one reactant, only one of the two Michaelis constants can approximate the equilibrium constant.

Confusion may also result from the definition of the Michaelis constant. Most enzymologists use an operational concept of the Michaelis constant as an experimentally measurable parameter for a system following typical "Michaelis-Menten" kinetics. This is preferred by the reviewer to the view taken by some recent workers that the true Michaelis constant is the dissociation constant for the enzyme-substrate combination.[6] This latter view agrees with the meaning that Michaelis and Menten originally ascribed to the constant, but this meaning is known to be incorrect. Perhaps the interesting point that the very useful relationship in question was clearly described by others before Michaelis [see Segal in (1)] makes less appropriate the use of Michaelis' interpretation.

Reaction Thermodynamics

Evaluation of thermodynamic parameters in enzymic catalyses is a field in its infancy. Lumry, Lang & Schellman [in (1)] give qualified and extensive

[6] Dixon & Webb (140) also consider this disparity and suggest that the term substrate constant, K_s, is analogous to an inhibitor constant, K_I, for the E-S dissociation constant. Another operational constant, the "exchange" constant is obtainable from exchange rate studies (152).

discussions; only brief comments will be made here. Present information warrants the tentative generalization that enzyme catalysis is characterized by a lowering of activation energy. The comparison between homogeneous catalysis and enzyme catalysis, however, is not simple, because different reaction paths may be involved. The picture described by Pauling (173) some 14 years ago that "the substrate molecule is attracted to the enzyme, and caused by the forces of attraction to assume the strained state which favors the chemical reaction, that is, the activation energy is decreased . . . " remains pertinent today.

Related to such a concept is the suggestion by Bernhard & Orgel (174) that alkyl phosphates inhibit esterases because the unstable enzyme-substrate transition complex is imitated by the inhibitor in one of its more stable complexes with the enzyme.

Information about entropies of binding and activation of enzyme reactions promises to give distinctive information about enzyme catalyses, but no generalizations have yet emerged. Lang & Schellman [in (1)] point out that the limited information indicative of unusual entropies of binding and activation gives strength to the idea that enzymes are more than particularly effective examples of rigid surface catalysis. More skilled experimentation in this area is needed.

Studies of Other Catalyses

Most of the information on enzyme catalysis and on heterogeneous catalysis has had little apparent relevance, and the fields have developed largely independently. Of interest is the recent discussion by Balandin (175) of his theory of "multiplet" catalysis in relation to enzyme action. This theory, a prominent part of which is simultaneous absorption at several points, has provided considerable stimulus over a period of years in heterogeneous catalysis studies [see (176)]. More pertinent to enzyme mechanisms appear to be studies of homogeneous catalysis in organic reactions, and a marked upswing in interest in such systems is evident.

Examples of promotion of rates by favorable steric arrangement of suitable groups are becoming numerous. Recent striking effects are those reported by Bender & Neveu (177) on the intramolecular catalysis of ester hydrolysis by carboxylate ions, and by Bruice & Sturtevant (178) on intramolecular participation of imidazoyl groups in ester and amide hydrolysis. Such results are indeed suggestive of the mode of possible participation of enzyme carboxyl and imidazoyl groups. A disturbing factor in the analogy to enzyme catalysis is the thermodynamic relationships. The limited data available for the corresponding intramolecular and closely similar intermolecular catalyses indicate that the major factor favoring intramolecular catalysis is the much smaller entropy of activation (177). As mentioned previously, enzyme reactions appear to be characterized by a lowering of energy of activation.

Other model system reactions might serve better to meet the thermodynamic requirements, although, at present, the necessary data are lacking. Steric compression effects have been suggested for marked increases in rate in examples of the Smiles Rearrangement (179) and in the so-called "gem-dimethyl" effect (180). An interesting effect of strain is cited by Cifonelli *et al.* (181), in which the marked lability to hydrolysis of the glycosides of 2:5-anhydro-L-arabinose is attributed to the strain introduced by the 2:5-anhydro ring.

Additional examples of possible interest include studies on the mode of participation of imidazole in catalysis [Jencks (182)] of cleavage of acetyl imidazole—the imidazole appears to act by removing a proton from the attacking reagent in the transition state. Wang (183) reports an interesting use of the ferric chelate of triethylenetetramine as a model for H_2O_2 decomposition; the activation energy of 6.6 kcal. was close to that of catalase, but the efficiency was poor. An interesting report of Cramer & Dietsche (184) describes asymetric synthesis and cleavage of mandelic acid esters in presence of cyclic Schardinger dextrins. Also, Cramer & Todd [see (185)] note that ADP readily forms inclusion compounds with cyclodextrins and that hydrolysis is promoted. Analogy of the behavior of the inclusion compounds to enzyme action is suggested.

Of the various interesting studies on electron transport, mention will be made only of the implications of the observations by Fraser, Sebera & Taube (186) on electron transfer through fumarate in the reaction of chromous ion with $(NH_3)_5Co^{++}—O—CO—CH=CH—CO—OCH_3$. Such studies are germane to suggestions that transport to metals in cytochromes may involve nonmetallic groupings. Theoretical treatments of molecular orbitals in enzyme catalysis and on 6-aminonicotinamide antagonism of DPN-dependent systems by Pullman & Pullman (187, 188) will be of interest to some readers.

Some Concluding Remarks

Present interest in, and the variety of approaches to, enzyme mechanisms promises vigorous development of the field. Some concepts are becoming clear, particularly the need for proper spatial orientation of reactive groups and the actual identification of participating groups of the enzyme. The importance of conformation changes in enzymes accompanying catalysis awaits elucidation. Probably, features other than proper orientation of substrate and enzyme groups are involved.

The reader will note that the reviewer favors mechanisms that can be visualized in terms of one key transition state. An hypothesis for consideration is that even apparently complex enzyme reactions involving three substrates can be visualized in this manner. Such would appear to be the simplest explanation and could logically involve need for only one spatial orientation on the enzyme for each participating substrate. Introduction of discrete, covalent intermediate stages may necessitate shifts in position of

bound substrates during catalysis and require that the enzyme be able to bind intermediates and other reactants so as to favor either of two discrete reactions, thus involving two key transition steps.

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PROTEOLYTIC ENZYMES[1,2]

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INTRODUCTION

For many years, proteolytic enzymes have been used as models for interpreting the relationship between protein structure and enzyme action. However, some doubt must always attach to the generality of results obtained with those enzymes whose physiological role and distribution are somewhat specialized, and it is therefore fortunate that similarities of mechanism, which extend to a wider class of hydrolases, are becoming apparent between proteinases of differing origin and specificity. The hope that such similarities of mechanism may be reflected in portions of the amino acid sequence in these enzymes remains a powerful stimulus to further study.

There is, therefore, sufficient evidence to attempt a classification of these enzymes based on mechanism of action, rather than origin, specificity, or physiological action. Though possibly premature, such a classification might be: (*a*) *Serine proteinases*, e.g. chymotrypsin, trypsin, elastase, thrombin, plasmin, and subtilisin, the active centres of which contain a serine residue that reacts uniquely with organophosphorous compounds. (*b*) *Thiol proteinases*, e.g., papain, ficin, bromelain; some cathepsins may be admitted to this group. (*c*) *Acid proteinases*, e.g., pepsin and rennin, are distinguished by their low optimum pH, which may imply a common mechanism. (*d*) *Metal proteinases*, e.g., carboxypeptidases, aminopeptidases and dipeptidases are generally exopeptidases; collagenase may be a metal endopeptidase. Insufficient is known about the properties of other proteinases to include them in this classification.

Certain approaches have proved fruitful with proteolytic enzymes. The special importance of purification has previously been emphasized (1). Kinetic studies with synthetic substrates have been very rewarding, but few valid generalizations can yet be made about the action of proteinases on proteins. Amino acid sequence studies have helped to elucidate the activation of zymogens and the environment of the active site, but there is growing agreement that a satisfactory explanation of enzyme activity and specificity may require knowledge of complete primary and secondary structure.

The preceding considerations have helped to shape this review. Wherever

[1] I have attempted to discuss relevant literature published between November 1, 1957, and October 1, 1959.

[2] The following abbreviations are used: FDNB for 1-fluoro-2,4-dinitrobenzene; Cbz- for carbobenzoxy-; DFP for di-isopropylphosphorofluoridate; IRC-50 or XE-64 for the corresponding Amberlite cation exchange resins.

possible, I have used the existence of previous reviews (1 to 7) as an excuse for limiting discussion, and comment is restricted to the endopeptidases. Even so, a number of useful contributions to our present knowledge must be discussed too briefly or not at all. Particularly ironic is the fact that the action of proteolytic enzymes on proteins is omitted from review, since here our ignorance is deepest.

THE SERINE PROTEINASES

Isolation and Purification

Ion-exchange chromatography has proved a powerful tool, both in analysis for purity and in purification of these enzymes and their zymogens. Chromatographic separations of α-chymotrypsinogen, α-chymotrypsin, trypsinogen, and thrombin have been previously reviewed (1, 5). Naughton & Sanger (8) report the purification of elastase on carboxymethyl cellulose and Miller (9) of prothrombin, Seegers *et al.* (10) of thrombin, and Gladner *et al.* of di-isopropyl-phosphoryl-thrombin (11) on IRC-50, and Spector & Ottesen (12) of subtilisin on carboxymethyl cellulose. In many of these cases it has been demonstrated that traces of active impurities are removed from preparations crystallized several times. Removal of such traces can be most important for studies of specificity or zymogen activation (e.g., 13) or in the use of proteolytic enzymes as tools in studies of amino acid sequence. Another hazard in the purification of proteinases is the possibility of proteolytic modification of the original enzyme during isolation. Chromatography of the zymogens of di-isopropyl-phosphoryl derivatives, at pH's distant from the activity optimum or in the presence of inhibitors (e.g., 14), represents attempts to overcome this problem. A promising new approach is suggested by the studies of Cole (15) on the chromatography of insulin in 8 *M* urea, and of Mitz & Schlueter (16) who demonstrate the stability of proteinases adsorbed to cellulose ion-exchange resins.

An important application of ion-exchange chromatography is the fractionation of bovine pancreatic juice by Keller, Cohen & Neurath (14). The chromatography on columns of diethylaminoethyl cellulose or XE-64 resins was satisfactorily compared with that of known mixtures of pancreatic enzymes, and the enzyme activities of the column fractions were assayed with synthetic substrates. Table I shows the protein composition of bovine pancreatic juice thus obtained. About 80 per cent of the protein consisted of known enzymes or zymogens, most of them proteolytic, which, wherever investigated, seemed identical with the crystalline enzymes obtained by conventional techniques. Thus we are reassured that the crystalline enzymes so commonly used are unlikely to be physiological artefacts, and the range of speculation about new enzymes likely to be found in high yield in pancreatic juice is effectively limited.

In an extension of this study, the same authors (17) have investigated the incorporation of radioactive cystine into the cationic proteins of the juice. They observed the same ratios of these proteins at different times of col-

TABLE I*

RELATIVE PROPORTIONS OF COMPONENTS OF BOVINE PANCREATIC JUICE

Component	Relative proportion (as percentage of total protein)
Proteolytic:	
Trypsinogen	14
Chymotrypsinogen-α	16
Chymotrypsinogen-B	16
Procarboxypeptidase=A	19
Procarboxypeptidase=B + Carboxypeptidase=B	7
Nucleolytic:	
Ribonuclease	2.4
Deoxyribonuclease	1.4
Amylolytic	<2
Lipolytic	Very low
Unidentified	10

* From Keller, Cohen & Neurath (14).

lection, and the specific S^{35} activities in trypsinogen and chymotrypsinogen were the same, suggesting equivalent mechanisms of synthesis and secretion for these two zymogens. Ribonuclease, however, was anomalous.

Crystalline bovine or porcine pancreatic elastase is clearly impure. Naughton & Sanger (8) have purified the porcine enzyme on carboxymethyl cellulose columns. The product differed markedly from trypsin or α-chymotrypsin in its specificity toward the B-chain of oxidised insulin and reacted stoichiometrically with DFP (18). By use of zone electrophoresis, Dvonch & Alburn (19) demonstrated the heterogeneity of the crystalline porcine enzyme and described purified fractions active towards elastin and haemoglobin; trypsin and α-chymotrypsin, in contrast, do not digest elastin. Lewis *et al.* (20) demonstrated the purity of elastase prepared electrophoretically and showed that it differs from trypsin or α-chymotrypsin in electrophoretic mobility, stability in acid solution, and in action on elastin. Somewhat anomalous reports by Czerkawski & Hall (21) of synergistic action between various "elastase" fractions separated by paper electrophoresis may reflect differences in the elastin substrate preparations used and shortcomings in the methods of assay. Furthermore, it seems possible that pankrin (22) and pankrinogen [Soejima & Shimura (23)] may be related to elastase or its zymogen.

Further criteria are now available for the purity of thrombin and prothrombin. After chromatography on XE-64 cation exchange resin, prothrombin is homogeneous by sedimentation analysis and immunological studies and contains one N-terminal alanine residue per mole [Miller (24)].

Pechet *et al.* (13) find that prothrombin purified by starch gel electrophoresis no longer shows "citrate activation," thus indicating that traces of activators have been removed. Another criterion of purity of thrombin is that the di-isopropyl-phosphoryl enzyme, purified on diethylaminoethyl cellulose columns, contains one mole phosphorus per mole enzyme [Gladner *et al.* (11)].

Progress in the field of fibrinolysis, reviewed by Sherry *et al.* (25), will clearly be assisted by purification of plasminogen and plasmin. Shulman, Alkjaersig & Sherry (26) demonstrate electrophoretic and ultracentrifugal inhomogeneity for even the best preparations, but Shulman (27) briefly reports further purification by ammonium sulphate fractionation or starch gel electrophoresis. The observation by Mounter & Shipley (28) of inhibition of plasmin by organophosphorus compounds suggests that purification of the zymogen in the presence of DFP may help to avoid activation during purification.

Ottesen & Schellman (29) have continued a series of careful studies on the purity of subtilisin. The broad specificity of this enzyme (30) and the possibility of mutants in the *Bacillus subtilis* source make such care desirable. The stoichiometry of reaction with DFP and with FDNB indicates a high degree of purity, but Spector & Ottesen (12) show that subtilisin can be separated from a similar *B. subtilis* proteinase on columns of carboxymethyl cellulose. Both appear to be homogeneous, behave similarly on electrophoresis or in the ultracentrifuge, and have similar activity towards ovalbumin, but differences can be detected in the kinetics of reaction with other substrates. This careful study points up the risk in identifying subtilisin with the crystalline *B. subtilis* protease isolated by Hagihara *et al.* (31). The isolation technique, crystalline form, action toward methyl butyrate, end groups, and apparent molecular weights of these enzymes appear to be very similar (32 to 35). Parallel investigations of activity toward the same substrate [e.g., the B-chain of oxidized insulin (30)], by use of comparative chromatotographic techniques (e.g., 18), might help to compare their specificity.

Specific adsorption of enzymes onto insoluble substrates, inhibitors, or antibodies may help to achieve high degrees of purity. Adsorption of elastase onto elastin has been reported previously (36). Erlanger (37) has reported adsorption of chymotrypsin or chymotrypsinogen to an insoluble inhibitor, while Stone & Williams (38) have described a cellulose column with adsorbed antibody which removed traces of subtilisin from carboxypeptidase preparations.

Specificity and Kinetics

Chymotrypsin and trypsin.—Specific substrates and inhibitors of chymotrypsin may be loosely defined as derivatives or analogues of L-tyrosine, L-tryptophan, and L-phenylalanine. Bixler & Niemann (39) neatly illustrate this with the L-substrate $R \cdot CO \cdot NH.(R' \cdot CH_2\text{-}) CH \cdot CO \cdot O \cdot CH_3$, where R is 4-pyridyl-1-oxide and R′ is phenyl, and with the benzoylnicotinylalanine derivative in which R and R′ are reversed. The former, N-nicotinylphenyl-

alanine ester, is hydrolysed much more rapidly than the substrate with a nicotinyl-1-oxide ring in the side chain. The bond hydrolysed may be peptide, ester, amide, hydroxamide, or hydrazide, but methylamides are hydrolysed only slowly, and Manning & Niemann (40) show that these act, in fact, as competitive inhibitors of other substrates. Similar results are presented by Lands & Niemann (41), who developed criteria for valid evaluation of competitive inhibition in such cases and thereby criticise some previous studies (42). D-Isomers of specific substrates have again been shown to be competitive inhibitors with an affinity for the enzyme generally, but not always, greater than that of the corresponding L-substrate [Manning & Niemann (43)]. With all such substrates so far examined, the results confirm that $K_m \sim k_2/k_1 = K_s$, and K_m is, therefore, a measure of enzyme-substrate affinity.[3] Table II summarizes some recent reports of kinetic constants for chymotrypsin.

TABLE II

KINETIC CONSTANTS FOR α-CHYMOTRYPSIN AT 25° AND PH 7.8 TO 8.0

Substrate	K_m M×10³	K_i M×10³	k_3 Sec⁻¹	Reference
Carbethoxy L-Tyr. methylamide		9.8		40
Carbethoxy D-Tyr. methylamide		23		40
Carbethoxy L-Tyr. amide	6.4		0.045	43
Carbethoxy D-Tyr. amide		21		43
Acetyl L-Tyr. amide	32		0.17	43
Acetyl L-Tyr. hydrazide	30		0.07	45
Acetyl L-Tyr. ethyl ester*	0.7		193	46
Acetyl L-Val. methyl ester	125		0.16	47
Acetyl Gly. methyl ester	10		0.008	48
n-Heptanoyl *o*-hydroxybenzoate	4		0.05	49
Ethyl D,L-lactate	110		0.04	50
Acetyl *p*-nitrophenate:				
Acylation	5.0		3.2	51, 52
Turnover	0.04		0.01	
Carbobenzoxy L-Tyr. *p*-nitrophenate†	0.03		~500	53

* 0.1 M $CaCl_2$.

† Temperature 30°.

Specific substrates of trypsin are derivatives or analogues of arginine or lysine. Izumiya & Uchio (54) have examined the kinetics of tryptic hydrolysis of the peptide amides, $Gly_n \cdot Lys\text{-}NH_2$, and find that splitting is maximal when n is 2 or 3. Ebata & Morita (55) find that ε-amino caproyl esters, amides, and peptides are split by trypsin at rates comparable with those of

[3] The kinetic nomenclature of Dixon & Webb (44) has been used throughout this review.

the corresponding benzoyl arginine esters and amides; this suggests that the "secondary peptide bond" in the latter is of little importance in determining specificity. A much weaker activity against tosyl homoarginine and ornithine esters [Elmore & Baines (56)] demonstrates that the length of the side chain is an important factor in substrate specificity. Nakatsu (57) has now shown that α-N-benzoyl canavaninamide, an analogue of α-N-benzoyl L-arginine amide, is split only slowly by trypsin.

Chymotrypsin has a weak activity toward esters of certain other acyl amino acids or analogues; these will be arbitrarily classified here as "low specificity substrates." Applewhite, Waite & Niemann (47) have studied acyl L-valine esters at pH 7.9, 25°. They experienced difficulty with more specific esters, such as acetyl L-phenylalanine glycolamide ester, because of the low concentrations of enzyme necessary when the reaction was followed in the pH-stat; they therefore recommend the use of the less rapidly hydrolysed substrates. Bixler & Niemann (58) show that the above difficulties result from errors in the dilution of low concentrations of enzyme by adsorption on volumetric glassware—another example of the hazards involved in handling dilute protein solutions at low ionic strength. However, as suggested by Tinoco (59), α-chymotrypsin polymerises at the concentrations used by Applewhite *et al.* (47), and this may add an additional complication to the kinetics. Indeed, the rate of hydrolysis of benzoyl glycine ethyl ester is not proportional to enzyme concentration at low ionic strength (67), and Martin & Niemann (61) attempt, rather speculatively, to draw conclusions from kinetic data about the polymerization. It would appear that these substrates are not ideal for assay of chymotryptic activity.

Some other substrates of chymotrypsin have extended our knowledge of its specificity. Of practical importance is the observation by Castañada-Agullo & Del Castillo (62) that the "trypsin substrate," α-N-benzoyl L-arginine ethyl ester, is slowly split by chymotrypsin, and inhibition studies make trypsin contamination an unlikely explanation for this effect. Hofstee (49) showed that chymotrypsin splits fatty acid esters of hydroxybenzoic acids and, from the effect of pH, concludes that hydrolysis of an acyl-enzyme cannot be the rate-limiting step. It is interesting that the optimal chain length for the acyl residue is C_7, permitting a configuration which might approximate to a six-membered ring. The hydrolysis of ethyl DL-lactate by chymotrypsin is inhibited by DFP and by β-phenyl propionate, so that Tinoco (50) appears justified in claiming this as a substrate for the enzyme. The ionization in the enzyme of a group of pK_a 6.7 appears to control the hydrolysis. A new use for chymotrypsin in suggested by Cohen & Altschul (63) in the asymmetric hydrolysis of acetamido malonyl ethyl ester.

The effects of salts on the kinetics of hydrolysis by trypsin and chymotrypsin have never been adequately explained. With chymotrypsin and acyl tyrosine amides (64, 65), K_m is unaffected by addition of salt, whereas k_3 increases; Kerr & Niemann (66) show a similar effect with an acyl tyrosine hydrazide. However, when benzoyl glycine or acetyl L-valine esters are the

substrates, a rapid increase in K_m is observed at low ionic strengths. This discrepancy may be linked to the aforementioned possibilities of polymerization at low ionic strengths. Specific activation by calcium ions in the hydrolysis of benzoyl glycine methyl ester is reported by Martin & Niemann (88) for α-chymotrypsin and by Lang *et al.* (67) for γ-chymotrypsin. These workers disagree as to whether the effect is exhibited in K_m or k_3, but in neither case is the rate proportional to enzyme concentration, and, with γ-chymotrypsin at least, autolysis may be involved. Jennings *et al.* (68) record the calcium activation of acyl tyrosine amide hydrolysis by chymotrypsin; this activation appears to depend on the nature and concentration of the buffer used. Despite these complex circumstances, I feel bound to accept the hypothesis of a specific binding of calcium ions to the enzyme, which may affect the kinetics and stability (see below) by altering secondary structure.

The effect of organic solvents can help to distinguish kinetic steps in a reaction, and Applewhite, Martin & Niemann (69) have used it to elucidate the hydrolysis of benzoyl glycine ethyl ester by chymotrypsin. With most solvents, other than alcohols, an increase in K_m is observed without any effect on k_3. These phenomena do not depend in any obvious fashion on the dielectric constant. The authors suggest that the substrate may partition between microscopically inhomogeneous water-solvent phases. Alteration of the configuration of the protein might also play a part at the low ionic strengths used, but perhaps these explanations represent different facets of the same physical phenomenon. Furthermore, in the presence of alcohols, acyl transfer reactions can occur; this additional complication makes it difficult to evaluate the studies by Canady & Laidler (70) or Stein & Laidler (71) on the chymotryptic hydrolysis of methyl hydrocinnamate in the presence of methanol.

With trypsin, a careful study by Castañada-Agullo & Del Castillo (72) of the hydrolysis of acyl arginine esters in the presence of low concentrations of organic solvents has brought to light some operational errors in an earlier work (73). Eschewing results at higher concentrations of alcohols, where transfer reactions may occur, they demonstrated a linear dependence of rate on the reciprocal of the dielectric constant. This is consistent with interaction between a positively charged substrate and a dipole in the active centre.

The reaction of chymotrypsin with *p*-nitrophenyl acetate conforms to the mechanism:

$$\mathrm{E{-}H + R{-}X \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \left[\begin{matrix}\mathrm{E{-}H}\\ \mathrm{R{-}X}\end{matrix}\right] \xrightarrow{k_3} E{-}R + X{-}H \xrightarrow[H_2O]{k_5} E{-}H + R{-}OH + X{-}H} \quad 1.$$

where E–H is enzyme; R is, in this case, acetyl; and X is nitrophenate. Spencer & Sturtevant (52) have extended previous fast reaction studies of this catalytic hydrolysis (51). They find that the ionization in the enzyme of a group of pK_a 7.4 at 25° controls k_5, whereas the corresponding pK_a for k_3 is 6.6 (51). By similar stopped-flow studies with trypsin and *p*-nitrophenyl

acetate, Stewart & Ouellet (74) show that Reaction 1 is obeyed, with apparent ionization constants of $pK_a \sim 7$ controlling k_3 and k_5. It is tempting to relate these ionization constants to that of an imidazole residue in the active centre of these enzymes, but Bruice & Schmir (75) recommend caution in identifying "kinetic" dissociation constants with true ionization constants. Stopped-flow studies (52) with the isolated acetyl-chymotrypsin of Balls & Wood (76) show that this must be identical with the acyl intermediate in Reaction 1. In contrast, Marini & Hess (77) claim that this acyl intermediate differs from the isolated acyl enzyme (76). A small lag in the zero-order hydrolysis at pH 8 and a greater reactivity of the acetyl group with hydroxylamine in the early stages of urea denaturation at pH 5.5 are reported for the Balls & Wood derivative. However, transfer of the acetyl group to hydroxylamine or to ethanol is markedly dependent on pH in the range 5 to 6 (78), and minor configurational changes in the protein during the isolation procedure might account for the results of Marini & Hess (77). Furthermore, Schonbaum, Nakamura & Bender (79), could detect no spectral differences between an acyl intermediate, as in Reaction 1, and the cinnamoyl enzyme isolated from the reaction of α-chymotrypsin with *o*-nitrophenyl cinnamate. In this connection, changes in the absorption of the acyl-enzyme at 245 mμ during incubation at pH 8 have been ascribed to the hydrolysis of an acyl imidazole (80). However, Spencer & Sturtevant (52) show that the rate of the spectral shift is only about 10 per cent of the rate of deacylation, measured under identical conditions, so that an acyl imidazole cannot be a direct intermediate in the hydrolysis. The weight of evidence seems to suggest that the acyl-enzyme is an ester of a unique serine residue [Oosterbaan & Van Adrichem (81)].

Are specific substrates hydrolysed via such acyl-enzyme intermediates? With trypsin, the turnover rates of a series of benzoyl arginine esters are identical (73), consistent with a rate-limiting deacylation step, but with chymotrypsin similar arguments cannot be used. Nevertheless, many of the transacylation reactions catalysed by chymotrypsin [(82)], or reactions of "virtual substrates" [(83)], suggest such a mechanism. Spencer & Sturtevant (52) have demonstrated that the acetyl enzyme formed during the hydrolysis of *p*-nitrophenyl acetate is unable to hydrolyse acetyl tyrosine ethyl ester. A more direct answer is suggested by the introduction of Cbz-L-tyrosine nitrophenyl ester by Martin, Golubow & Axelrod (53) as a substrate for many proteolytic enzymes. Gutfreund & Hammond (84) show that this substrate gives the "burst" of nitrophenol which is characteristic of *p*-nitrophenyl acetate hydrolysis, indicating that with this analogue of N-acetyl L-tyrosine ethyl ester, an acyl enzyme must be formed. Since the turnover rates of other acyl tyrosine substrates are less than that of the nitrophenyl ester (Table II), it appears that, with chymotrypsin, the acylation step is generally rate-limiting.

A new light is shed on the mechanism by observations with competitive inhibitors of chymotrypsin and low-specificity substrates. Awad (85) ob-

served that indole and β-phenyl propionate act as classical competitive inhibitors of the acylation of chymotrypsin by *p*-nitrophenyl acetate (k_3), but activate the deacylation (k_5) by formation of acyl-enzyme-inhibitor complexes, the K_i of which is of the same order as for the free enzyme-inhibitor complex. He concludes that the small size of the acetyl group allows binding of indole at the "specificity site," thereby stimulating hydrolysis of the adjacent acyl group. Some observations from Niemann's laboratory on the hydrolysis of benzoyl glycine methyl ester by chymotrypsin support this hypothesis. The effect of indole is explained by Applewhite, Martin & Niemann (69) as formation of a ternary complex of enzyme, substrate, and inhibitor that can still hydrolyse. A similar ternary complex of enzyme and two substrate molecules, which hydrolyses faster than the normal Michaelis complex, is adduced by Wolf & Niemann (48) to explain the activation of the hydrolysis of benzoyl glycine ethyl ester in the presence of excess substrate. Furthermore, a ternary complex of benzoyl glycine methyl ester, chymotrypsin, and the inhibitor acetyl L-tryptophan methylamide can also yield hydrolysis products, according to Applewhite & Niemann (86). This behaviour of the glycine ester depends on the absence of an α-side chain, since Applewhite & Niemann (86) find no evidence of a ternary complex with benzoyl L-valine ethyl ester as substrate in similar circumstances.

Such observations focus attention on an often ignored aspect of hydrolysis by chymotrypsin, namely, that the rate of hydrolysis of the acetyl tyrosyl enzyme must be at least 16,000 times faster than that of the acetyl enzyme (Table II). The simplest explanation of all these results seems to be that binding of the substrate at the "specificity site" involves a configurational adaptation of the enzyme which brings "activating groups" into favourable juxtaposition.[4]

Other serine enzymes.—The specificity of elastase toward the B-chain of oxidised insulin is described by Naughton & Sanger (8). Its action is quite different from either trypsin or chymotrypsin (89), and, though no marked specificity can be deduced, a preference for hydrolysis near aromatic or long-chain aliphatic amino acids seems indicated. The brief observations of Yu *et al.* (90) on the action of elastase on some amino acid and dipeptide derivatives are not inconsistent with such a specificity.

Thrombin hydrolyses tosyl arginine methyl ester and lysine ethyl ester. Scheraga *et al.* (91) have shown that the arginine ester is split more rapidly by thrombin, trypsin, and plasmin, but, whereas with plasmin the differences between the substrates appear mainly in K_m, with trypsin and thrombin the lysine ester shows a lower k_3. The effect of pH on the hydrolysis of α-N-tosyl arginine methyl ester by thrombin and trypsin has been analyzed by Ronwin (92), but the results appear complex. Many studies, not detailed here, show that the action of thrombin on its physiological substrate, fibrinogen, involves specific cleavages at two arginine residues near the N-

[4] Neurath & Hartley (87) discuss the mechanism of hydrolysis by chymotrypsin and trypsin in greater detail.

terminus of the molecule and liberates two peptides containing 19 and 21 residues, respectively. A staphylococcal "coagulase" is shown by Haughton & Duthie (93) to resemble thrombin in its specificity.

Sherry, Fletcher & Alkjaersig (25) have recently reviewed plasmin and fibrinolysis. Differences between plasmin, trypsin, and thrombin in the hydrolysis of arginine and lysine esters have been described above, but Lassen (94) suggests that part of the lysine esterase activity is associated with impurities in plasmin. Further specificity studies with plasmin are clearly desirable, perhaps with peptides of known amino acid sequence; purification of the enzyme would be a desirable first step. With subtilisin, Spector & Ottesen (12) have clearly demonstrated the necessity for careful purification, since specificity differences were detected between enzymes which were otherwise almost identical.

Activation of Zymogens: End Groups

Except for subtilisin, zymogen precursors which are activated by specific proteolytic cleavages have been reported for all the serine proteinases. The chemical changes accompanying the activation of trypsinogen and α-chymotrypsinogen have been authoritatively reviewed by Neurath (5). In α-chymotrypsinogen, activity appears coincident with the splitting of an Arg·Ileu bond near the N-terminal end of the single peptide chain; up to three further specific chymotryptic cleavages can occur, leading to a family of neo-chymotrypsins and neo-chymotrypsinogens. Recent reports of the C-terminal residue in α-chymotrypsinogen complete our knowledge of its end groups and resolve previous controversy. In the native zymogen, this C-terminal group does not react with carboxypeptidase-A, but Pechère *et al.* (95), using carboxypeptidase-A in the presence of 8*M* urea, find a C-terminal asparagine in S-sulfo α-chymotrypsinogen. Meedom (96) reports similar findings with performic-oxidised α-chymotrypsinogen. Rovery *et al.* (97) show that this C-terminal asparagine is liberated by carboxypeptidase as the zymogen is denatured by urea, but surprisingly report that, after removal of urea, the "renatured" zymogen remains sensitive to carboxypeptidase. The rate of liberation of C-terminal residues by carboxypeptidase suggests the C-terminal sequences: —Try·(Ser, Ala)·Val·Thr·Leu·Ala·Asp-NH_2 (95), or —(Ser, Glu)·(Ala, Val, Thr)·Leu·Ala·Asp-NH_2 (96), or —Thr·Val·Leu·Ala·Asp-NH_2 (97). During the activation, changes occur in the secondary and tertiary structure of chymotrypsinogen and are reflected in differences in optical rotation or in spectral shifts (98). Chervenka (99) shows that these spectral shifts are characteristic of tyrosine residues, whereas in denaturation spectral changes in all chromophoric groups are indicated. In the denaturation of trypsin, Smillie (100) finds similar spectral changes, but there is no spectral shift during the activation of trypsinogen. This would suggest that the structural alterations after activation involve a limited part of the molecule and that some configurations may be relatively little affected. This immediately brings to mind the suggestion of Vaslow & Doherty (101) that the

"specificity site" may be intact in the zymogen. Orientation of the activating groups must, however, be brought about by the structural change.

Little is known about the activation of chymotrypsinogen-B, but Kassell (102) briefly reports that tryptic activation parallels the appearance of a C-terminal arginine residue, whereas the zymogen itself possesses C-terminal leucine and asparagine. She suggests that a series of neo-chymotrypsinogen-B's may exist. Neurath *et al.* (7) have again been unable to obtain a C-terminal residue from trypsinogen or S-sulfo trypsinogen by reaction with carboxypeptidase-A or -B.

Many different methods have been used to activate prothrombin (103), but impurities in prothrombin preparations may be the cause of some confusion. Pechet *et al.* (13) state that purified prothrombin is no longer activated by citrate, thrombin, or papain, but that trypsin still activates. Milstone (104) describes the purification of plasma thrombokinase, which may assist such studies. Activation by thromboplastin is assumed, by Ronwin (105), to lead to an inactive "thrombinogen" precursor, which is, in turn, activated by thrombin or other enzymes. In the same reaction, Seegers *et al.* (106) find an inactive intermediate with N-terminal proline; they suggest that the intermediate is later split to give thrombin with N-terminal glutamic acid! In contrast, Miller (24) finds no evidence for an inactive intermediate. By use of chromatographically pure zymogen, he finds that thrombin activity appears at the same rate as the N-terminal threonine of the thrombin finally isolated and that the rates of disappearance of prothrombin and of appearance of thrombin activity are the same! Studies of the precursors of plasmin [Alkjaersig *et al.* (107)] and elastase [Grant & Robbins (36)] are handicapped by lack of pure materials.

Primary Structure and Chemical Modification

Amino acid sequence.—Comparison of the complete amino acid sequences in trypsin and chymotrypsin will be the pleasant task of some future author of this chapter. Active investigation is still in progress, and the fragmentary information presently available has been recently reviewed, (2). Only a brief summary of the present situation will therefore be given here.

Rovery *et al.* (97), by terminal group analysis of the three peptide chains from performic-oxidised α-chymotrypsin, have confirmed that their sequence in chymotrypsinogen is A—B—C. Hartley (108 to 110) has continued studies of the large B-chain, which contains both histidines and the reactive serine residue. Fourteen soluble peptides and an insoluble "core" were isolated from a chymotryptic digest of the B-chain, and the amino acid sequences were determined. Nine of these peptides represent unique sequences which, together with the sequence of the A-chain (111), the end-group determinations discussed above, and the di-isopropyl-phosphoryl-serine sequence of Oosterbaan *et al.* (112), account for 108 of the 242 residues in the zymogen. The insoluble "core" appears to be an acidic peptide of around 100 residues; it contains no basic amino acids apart from the two histidine residues and possibly

contains the reactive serine residue. However, studies with performic-oxidised chymotrypsin are necessarily limited because of the unknown properties of the oxidised tryptophan residues. Alternative methods of cleaving the disulfide bridges in proteins, reviewed previously (2), are therefore very welcome.

Šorm and his colleagues have continued comparative studies of di- and tripeptides from partial acid hydrolysates of oxidised di-isopropyl-phosphoryl-trypsin and α-chymotrypsinogen (113, 114). Meloun *et al.* (115) report some small basic peptides, and Keil & Šorm (116) some peptides containing cysteic acid. From chymotrypsinogen, the latter authors have obtained unique di- or tripeptide sequences accounting for all ten half-cystine residues in the protein. A comparison (113) of about 59 small peptides from di-isopropyl-phosphoryl-trypsin with similar fractions from chymotrypsinogen shows that 35 of the trypsin peptides have exact counterparts in chymotrypsinogen. Gaboleteau & Desnuelle (117) report the sequence of some peptides from trypsin autolysates. There is a very strong presumption that portions of the amino acid sequence in trypsin and chymotrypsin may be identical.

Organophosphorus compounds.—Specific stoichiometric inhibition by organophosphorus compounds is the definition adopted herein for a "serine" enzyme. Ooms *et al.* (118) have compared the rates of reaction of some such enzymes with dialkyl *p*-nitrophenyl phosphates. It is generally accepted that a serine residue is the ultimate site of reaction, but there has been controversy as to whether a phosphorylated histidine might be an intermediate (119). One argument is that, on storage, dialkylphosphoryl-cholinesterase loses its ability to reactivate by treatment with oximes. This progressive loss of "reactivatability" has been ascribed to phosphoryl transfer from histidine to serine in the active centre (120). Green & Nicholls (121) show that dialkyl-phosphoryl-chymotrypsin can be fully reactivated by oximes and assume a phosphoryl transfer from serine to histidine to explain the pH dependence of the reaction with oximes. However, a more convincing explanation for the "ageing" phenomenon in di-isopropyl-phosphoryl-pseudocholinesterase is offered by Berends *et al.* (122), who show that, on storage, the di-isopropyl-phosphoryl group is hydrolysed to a monoisopropyl-phosphoryl group, which no longer reacts with oximes. Lee & Turnbull (123) demonstrate similar lability of a phenyl group in diphenyl phosphoryl chymotrypsin.

Wherever they have been investigated, similarities of the sequence surrounding the di-isopropyl-phosphoryl-serine have been found in all "serine" enzymes. A recent addition to the list is di-isopropyl-phosphoryl-pseudocholinesterase [Jansz *et al.* (124)]. In performic-oxidised di-isopropyl-phosphoryl-trypsin, Dixon, Kauffman & Neurath (125) find this sequence to be Asp·Ser·$CySO_3H$·Glu·Gly·Gly·Asp·Ser(DIP)·Gly·Pro·Val·$CySO_3H$·Ser·Gly·Lys. Speculation about the role of the two half-cystine residues must await further structural studies. The similar sequence from di-isopropyl-phosphoryl-chymotrypsin: Gly·Asp·Ser(DIP)·Gly·Gly·Pro·Leu, reported by Oosterbaan *et al.* (112) has been isolated by mild enzymic hydrolysis and studied by careful quantitative techniques. Furthermore, a

similar peptide from acetyl-chymotrypsin is reported by Oosterbaan & Van Adrichem (81). It is therefore possible that the explanation for discrepancies between this sequence and others (2) obtained by partial acid hydrolysis of the dialkylphosphoryl enzyme may lie in the more violent conditions of degradation used in the latter studies. One hopes that this conflict will be resolved.

Bovine pancreatic elastase is inhibited by DFP [Naughton & Sanger (8)], and Hartley, Naughton & Sanger (18) have shown that the amino acid sequence around the di-isopropyl-phosphoryl-serine is similar to that in trypsin and chymotrypsin. They use comparative paper electrophoresis of radioactive peptides from partial acid hydrolysates of the three di-isopropyl-phosphoryl-enzymes and conclude that a sequence Gly·Asp·Ser (DIP)·Gly is probably common to each. Mounter & Shipley (28) record that plasmin is inhibited by DFP, whereas plasminogen is not. Use of a series of organophosphorus inhibitors with trypsin and plasmin suggests the possibility of differential inhibition of these enzymes. A crystalline *Bacillus subtilis* proteinase also reacts stoichiometrically with DFP, and Matsubara (126) finds that, here also, serine is the site of action.

Active fragments.—In a sense, α-, β-, γ-, δ-, and the neo-chymotrypsins are "active fragments" of π-chymotrypsin (5), but the "junk hypothesis" presupposes that much greater degrees of fragmentation can produce active enzymes. With proteolytic enzymes, as Liener (127) and Wootton & Hess (128) point out, digestion of denatured impurities in the starting preparation can lead to a spurious increase in specific activity. Some "active fragments" are reported by Hess & Wainfan (129) in dialysates of autolysed trypsin preparations, but these were not observed when pure trypsin was used. A preliminary communication by Viswanatha (130) expands some claims for an active fragment obtained by peptic digestion of an acetylated trypsinogen preparation. This fragment has been reported to be homogeneous in the ultracentrifuge and about a quarter the size of native trypsin (131). Reaction with carboxypeptidase-A is reported to inactivate the preparation when two amino acids are liberated, but aminopeptidase apparently removes 16 to 18 amino acids without destroying activity. These claims rest on the accuracy of assay of the specific trypsin activity and the absence of high molecular weight impurities in the preparations. The cautious note sounded by this reviewer will, perhaps, be forgiven in view of the importance which these fragments would have in our study of enzyme activity.

Denaturation.—Trypsinogen and chymotrypsinogen offer interesting models for study of the phenomenon of reversible denaturation. Stauff & Rasper (132) have used changes in refractive increment, pH, volume, sedimentation constant, and light-scattering to follow the reversible heat denaturation of α-chymotrypsinogen at pH 3. On heating, a slow change in these properties is followed, at about 40°, by a sharp reversible jump, which they ascribe predominantly to changes in water of hydration. Wilcox (133) shows that deuterium exchange increases after such reversible denaturation. Wilcox & Manning (134) have studied the properties of the zymogen in urea and

show that the abnormal ionization of two of the four tyrosine residues vanishes in 8*M* urea, and that this, the deuterium exchange properties, and others are reversible on dilution. However, Balls and his colleagues (135, 137) have observed that anions, particularly sulphate, decrease the reversibility of urea or heat denaturation of α-chymotrypsin. It seems possible that this anion effect may be connected with removal of calcium ions, since Chervenka (99) shows that calcium ions prevent spectral changes with δ-chymotrypsin in urea, and Damaskus & Bradford (138) show the powerful stabilizing action of calcium in aqueous solutions. The hypothesis that N→O acyl shifts may be involved in reversible acid denaturation seems less likely from the studies of Smillie & Neurath (139) with trypsin and anhydrous formic acid.

Model Compounds and Mechanism

With our present imperfect knowledge of enzyme structure, the merit of model compounds must be to suggest further experiments. The reaction of a postulated histidine in the active centre with *p*-nitrophenyl acetate has been the favourite objective of such models, but it is important to note that there is no direct evidence, as is available with ribonuclease (140) for the participation of such a residue. Nevertheless, model experiments with imidazole compounds (141) have some interesting features.

Some of the most attractive models demonstrate intramolecular catalysis. Thus *p*-nitrophenyl-α(4-imidazolyl) butyrate is a model for *p*-nitrophenyl acetate bound in close proximity to an imidazole ring [Bruice & Sturtevant (142)]. The effect of pH on the hydrolysis of this compound indicates formation of an acyl imidazole intermediate at a rate ($k = 3{,}3$ sec^{-1}, 25°, $H^+ = 0$) almost the same as that for the comparable acylation of chymotrypsin by *p*-nitrophenyl acetate [$k_3 = 4.0$ sec^{-1}, 25°, $H^+ = 0$ (51)]. Schmir & Bruice (143) and Bruice & Sturtevant (144) present similar models.

All these authors point out, however, that a serine residue must be presumed to play a part in the enzymic catalysis, and mechanisms in which the serine hydroxyl group forms hydrogen bonds to an imidazole nitrogen (145), with the option of acyl transfer between serine and histidine (121), are still in vogue. To this reviewer, however, such mechanisms seem oversimplified, since, in all serine enzymes, a negatively charged aspartic or glutamic residue occurs N-terminal to the reactive serine. This might influence the catalysis. The mechanism of Rydon (146), involving oxazoline ring formation by the serine residue, conversely fails to explain much of the evidence pointing to histidine. Mechanisms based on the carboxyl group and serine alone fail to explain why all activities of the enzymes vanish when the secondary structure is disrupted. A further, more serious, drawback of these mechanisms is that they fail to allow for the profound effect of substrate specificity on all steps in the catalysis. Perhaps the "specificity site" may prove to be the *deus ex machina*, which resolves our difficulties with the mechanism.

TABLE III

KINETIC CONSTANTS FOR PAPAIN AND FICIN

Substrate*	Enzyme	Temperature °C.	pH	K_m M×10³	k_3 sec⁻¹	k_3 (relative†)	Reference
BAEE	Papain	37	5.5	1.9	12.0	[1.00]	151
BAEE	Papain	25	5.5	2.2	8.8	—	151
BAEE	Ficin	25	6.1	2.5	—	[1.00]	154
BAA	Papain	38	5.2	4.0	11.0	0.92	150
BAA	Ficin	25	6.1	4.8	—	0.90	154
BGA	Papain	38	6.0	16.0	0.6	0.05	152
BGA	Ficin	25	6.1	13.0	—	0.04	154
BGEE	Ficin	25	6.1	4.8	—	1.40	154
CHA	Papain	38	6.2	13.0	4.0	0.33	152
CHA	Papain	38	5.2	2.0	0.5	0.04	152
CGG	Papain	38	6.0	28.	0.08	0.01	152

* The following abbreviations are used: BAA for α-N-benzoyl L-arginine amide; BAEE for α-N-benzoyl L-arginine ethyl ester; BGA for benzoyl glycine amide; BGEE for benzoyl glycine ethyl ester; CGG for carbobenzoxy glycyl glycine; CHA for α-N-carbobenzoxy L-histidinamide.

† k_3 Relative to that for BAEE. For papain, rates compared at 38°; for ficin at 25°

THIOL PROTEASES

PAPAIN AND FICIN

Smith and his colleagues have established papain as a most interesting model enzyme, and work with other plant proteolytic enzymes, notably ficin, suggests that papain may represent a class of "thiol" proteinases. Excellent recent reviews are available to document and augment the following brief outline (2, 6).

Purification.—Papain is pure by criteria of sedimentation, electrophoresis, ion-exchange chromatography, antigenic properties, and amino acid analysis. The activity is particularly sensitive to oxidation and to heavy metals, but the enzyme can be safely stored as the crystalline mercury derivative and assayed in the presence of reducing agents and metal-chelating agents. Multi-bed ion-exchange columns, by which active ion-free papain can be prepared, have been described by Finkle & Smith (147). Less data are available for the purity of ficin, but Cohen (148) reports that the crystalline enzyme is relatively homogeneous in the ultracentrifuge and on electrophoresis.

Kinetics.—The broad specificity of papain is shown, for example, against the A-chain of oxidized insulin (149), but, here again, synthetic substrates have been most helpful in elucidating the mechanism. Table III shows some kinetic constants for these two enzymes. The magnitude and similarity of

these constants for a range of substrates suggests that ficin and papain have a similar specificity and mechanism of action.

The effect of pH on the hydrolysis of these substrates has been an important factor in elucidating the mechanism of action. With papain and α-N-benzoyl L-arginine amide, Stockell & Smith (150) observed a rather peculiar pH-activity relationship but concluded, from similarities in the effect of pH on k_3 and K_m, that K_m approximated k_3/k_1. In contrast, with papain and α-N-benzoyl L-arginine ethyl ester, Smith & Parker (151) showed that the variation of k_3 with pH is described by an ionization pK_a of 3.7 at 35°, while K_m passes through a minimum at about pH 6. Furthermore, impurities in the benzoyl arginine ethyl ester (from which the amide is prepared) gave anomalous kinetics resembling the situation with the amide. It seems probable that reinvestigation of the kinetics of α-N-benzoyl L-arginine amide would lead to a situation resembling that with the ethyl ester, where there is no positive kinetic evidence that $K_m = k_3/k_1$.

Because of the insolubility of three other substrates of papain, Smith, Chavré & Parker (152) were unable to measure K_m and k_3 over a wide pH range, but, with benzoyl glycinamide, the effect of pH appeared to resemble that with the benzoyl arginine ethyl ester. With the substrate, carbobenzoxyglycylglycine, however, an increase in rate below pH 5 indicates that a charged carboxyl group in the substrate is inimical to the hydrolysis. With carbobenzoxy L-histidine amide, both k_3 and K_m increased rapidly between pH 5.5 and 7.0. This effect will be discussed later. However, if it is arbitrarily assumed that, with all substrates $K_m = k_3/k_1$, then the effects of pH might suggest the ionisation of groups in the enzyme of $pK_a \sim 4.3$ and ~ 8.0 which control k_1. In the cases studied, there appears to be considerable variation in the apparent heats of ionisation of such groups, but, with α-N-benzoyl L-arginine ethyl ester, the values would be 0 and 5.1 kcal./mole, respectively, at 0°.

With ficin, previous studies by Bernhard & Gutfreund (153) on the hydrolysis of α-N-benzoyl L-arginine ethyl ester had shown that, as with papain, k_3 is constant above pH 5 and decreases below this, suggesting an ionising group ($pK_a \sim 4.3$) in the enzyme but K_m was practically independent of pH from 3.8 to 6.5. Hammond & Gutfreund (154) showed that the rates of hydrolysis of four substrates of ficin (α-N-benzoyl L-arginine ethyl ester and the amide; benzoyl glycine ethyl ester and the amide), are identically affected by pH; they conclude that groups in the enzyme ionising at pK_a 4.3 and 8.4 are responsible. If their results be compared with those of Smith & Parker (151) for papain, it would appear that the ionisation at pH 8.4 affects mainly K_m, whereas the ionisation at pH 4.3 is manifested in both K_m and k_3.

Hammond & Gutfreund (154) have studied the effect of isopropanol on the hydrolysis of some substrates of ficin. With benzoyl glycine ethyl ester, the effect of isopropanol is entirely consistent with competitive inhibition, but, with benzoyl arginine ethyl ester the main effect is a decrease in k_3. This

decrease was shown to depend on dielectric constant in a fashion consistent with interaction between the positively charged substrate and a positive charge in the enzyme. Some preliminary results are also available on the effects of temperature and of salts on the catalysis by ficin (154). Increase of ionic strength appears to affect mainly k_3, but some specific ion effects are indicated. The effects of temperature obey the usual Arrhenius equations and suggest that earlier anomalies with papain (150) were attributable to impurities in the substrate.

Mechanism of action.—A thiol-ester intermediate has previously been assumed in catalysis by papain (150) or ficin (152). This would help to explain why the turnover rates of the benzoyl arginine amide and ester are almost identical (Table III) and why papain catalyses acyl transfer reactions with such ease (155). Smith (156) has proposed an extension of this mechanism in which the native enzyme is assumed to contain an internal ester of the reactive thiol and a side chain or terminal carboxyl group (Figure 1).

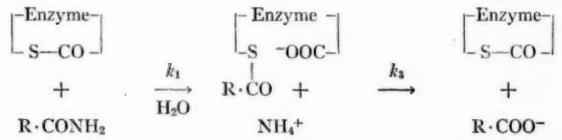

FIG. 1. Mechanism of hydrolysis by papain (156).

This might help explain the unusual chemical reactivity of the "thiol" group, but it is difficult to see how this mechanism could explain the kinetic data mentioned above. If the ionising groups of pK_a 4.3 and 8.0, which control the catalysis in both ficin and papain, are assumed to be carboxyl and thiol, respectively (156), then k_1 should be independent of pH, and k_3 should be controlled only by the carboxyl ionisation. If $K_m = k_3/k_1$, as the mechanism assumes (156), no effect on K_m should be observed above pH 5, since the thiol ester cannot ionise. In fact, the kinetic data suggest that the ionisation of a carboxyl group increases k_3 and decreases K_m, while the ionisation of a thiol group of pK_a 8.0 increases K_m but does not affect k_3.

Hammond & Gutfreund (154) assume a three-step mechanism (Reaction 1) in which k_5, the hydrolysis of a thiol ester, is rate-limiting in the case of benzoyl arginine ethyl ester and amide and benzoyl glycine ester, but k_3, the acylation step, is rate-limiting with benzoyl glycine amide (Table III). The ionising groups which control the rate are assumed to be carboxyl and amino, the latter because of the effect of dielectric constant on k_5. However, if an amino group were present in the active site, it is difficult to see how its ionisation around pH 7 to 8 could have no effect on the rate of hydrolysis of a nearby thiol ester (k_5), which the kinetics would suggest. This difficulty does not arise if we ascribe the ionisation, pK_a 8.0, to the thiol group.

The simplest hypothesis would seem to be that an unionised thiol group

is necessary for formation of the acyl enzyme and an ionised carboxyl group or groups for both formation and hydrolysis of the acyl enzyme. This carboxyl group could be spatially remote from the substrate, yet control the configuration of the active centre. Otherwise, with a negative charge in the active centre, it is difficult to see how the magnitude and effect of pH on K_m for the positively charged benzoyl arginine amide and the neutral benzoyl glycine amide could be so similar (154). Furthermore, if it be assumed that positively charged substrates are optimal for papain, we must explain the large decrease in k_3 for carbobenzoxy histidine amide as the imidazole residue gains a positive charge (152). This could be caused by internal catalysis of the deacylation by the uncharged imidazole side chain in the substrate, such as in the model compound of Bruice & Sturtevant (142).

Further perusal of Table III emphasizes how difficult it is to define the specificity of ficin or, indeed, of any enzyme. If we assume, for the sake of simplicity, that three constants, K_m (binding), k_3 (acylation), and k_5 (deacylation), can describe the kinetics of ficin, then structural alterations in the substrate can affect each of these, and it does not follow that changes that favour one step will necessarily favour another. Even if K_m be excluded from consideration, the rate-limiting step may alter from one substrate to another. Thus, comparison of the hydrolysis of benzoyl arginine and benzoyl glycine amides by ficin might suggest that positively charged substrates are split most rapidly by the enzyme. Comparison of the hydrolysis of the corresponding ethyl esters of benzoyl arginine and benzoyl glycine, would contradict this. Undoubtedly, we are comparing different rate-limiting steps, but such observations are not mere sophistication, since we have similar difficulty in defining the specificity of papain against peptide substrates on even the most pragmatic level.

Primary structure and chemical modification.—Papain is a single peptide chain containing 180 residues with N-terminal isoleucine (6). Six cysteic acid residues are found in performic-oxidised papain, and six thiol groups react with *p*-chloromercuribenzoate in the alcohol-denatured enzyme. Six cysteine residues are therefore present, but the nature of two additional sulfur atoms, indicated by elementary analysis, is unknown. One thiol group is uniquely reactive and can be selectively labelled with C^{14}-iodoacetamide (157). There is little doubt that this thiol group forms part of the active centre in papain.

Smith and his colleagues report considerable progress toward determination of the amino acid sequence in papain. Tryptic (157) or chymotryptic (158) digests of the oxidised enzyme yield soluble peptides and an insoluble "core." From such a "core" from papain labelled with C^{14}-iodoacetamide, Kimmel *et al.*, (157) have isolated a peptide of 30 or 40 residues; it had N-terminal glycine and contained the isotopically labelled "active" cysteine. The soluble peptides from the tryptic or chymotryptic digests of oxidised papain have been fractionated, and their amino acid compositions and, in some cases, their sequence have been determined. Kimmel *et al.* (159) report

that the composition of these peptides accounts for 110 of the 180 residues in the chain, and that "overlaps" between the tryptic and chymotryptic peptides allow partial sequences to be allocated—31 residues from the N-terminus, 25 from the C-terminus, and 33 from the center of the chain; further results are eagerly awaited.

Shields, Hill & Smith (160) discuss guanidinated mercuripapain. Seven of the eight lysine residues reacted with O-methyl isourea, and the crystalline product had the same specific activity and specificity as papain itself. No reaction with the remaining lysine, the α-amino group, or any other residue could be detected. The remaining lysine residue did not react with nitrous acid, nor was it attacked by trypsin, since a tryptic digest gave only free arginine after reaction with carboxypeptidase-B, but it did react with FDNB. Nitrous acid rapidly inactivated papain itself, and the guanidinated papain more slowly, apparently by reaction with amino and tyrosyl groups. Cohen (148) reports that 40 per cent of the amino groups in ficin can be acetylated without loss of activity.

Active fragments.—Although reviewed previously (2, 6), reference must be made to the degradation of mercuripapain with leucine aminopeptidase [Hill & Smith (161)]. Careful balance sheets were drawn up between the amino acids liberated and the composition of the remaining fragments. Up to 125 residues could be removed without loss of activity or marked change in specificity of the preparation, and fragments of an average length of 108 residues, which possessed this activity, were isolated. Controls established that the degradation was neither that of inactive contaminants nor of the leucine aminopeptidase. Shields *et al.* (160) showed that guanidinated mercuripapain may be similarly degraded and that some homoarginine residues are removed in the process. We must conclude that a large N-terminal part of the sequence in papain does not contribute directly to the activity or specificity.

Denaturation.—Despite the remarkable liberties which may be taken with the papain molecule without destruction of its activity, some part at least of the secondary structure remains essential. Hill, Schwartz & Smith (162) have shown that papain is inactive in 8M urea or 4M guanidine. Changes in optical rotation are associated with the inactivation, and the changes are, in part, reversible. Their data for inactivation are, however, puzzling. Inactivation is detected at low concentrations of urea, considerably below those required to effect changes in optical rotation. Traces of metal ions in the urea might swamp the amounts of cysteine activator and chelating agent used in the assay and could lead to the observed effect.

Other Plant Proteinases

Murachi & Neurath (163) report the purification of bromelain from the stems of pineapples by chromatography on XE-64 resins. The purified enzyme, strongly inhibited by mercuric ions, may be reactivated by cysteine. Some preliminary studies of the specificity suggest that it may be similar to,

but not identical with, that of papain. In glucagon, preferential splitting of either —Arg·Ala— or the adjacent —Ala·Glu(NH_2)— bonds was observed. No inhibition by DFP could be detected, but Heinicke & Mori (164) report that the crude enzyme is inhibited by the organophosphorus compound. They conclude that reaction is with an active thiol group, but the conditions of assay used might allow alternative interpretations. Arcus (165) finds a "thiol" proteinase, actinidin, in the fruit of the Chinese gooseberry.

"ACID" PROTEINASES

Pepsin

Recent reviews on pepsin have dealt with its structure and the activation of its zymogen (2, 5) and are the starting point for the following brief outline.

Structure.—Pepsinogen is a single chain of 400 residues with N-terminal isoleucine and C-terminal alanine. Specific proteolytic cleavages, autocatalyzed in acid, yield pepsin with N-terminal leucine, a "pepsin inhibitor" of 29 residues with N-terminal leucine, and some smaller peptides. The amino acid compositions of pepsinogen and the pepsin inhibitor peptide are known (5), and Blumenfeld & Perlmann (60) have recently provided us with a careful amino acid analysis of crystalline pepsin. Although the protein was apparently homogeneous in the ultracentrifuge or on electrophoresis, amino acid analysis indicated some impurity, particularly with respect to the hydroxyproline content. The authors suggest that this may indicate a family of pepsins of similar properties but slightly differing structures. They found 341 amino acid residues in the molecule, among them about 35 free acidic residues and only 4 basic residues. Previous results (136) have shown that a phosphate di-ester bridge contributes to the stability of the enzyme but is not essential for activity. Three disulphide bridges are indicated from the work of Blumenfeld & Perlmann (60). Pairent *et al.* (166) have commenced studies on the tryptic digestion of pepsin.

Activity.—Kinetic studies with pepsin are hampered by the lack of good synthetic substrates. Dipeptides of aromatic acids are among the best, but Neumann *et al.* (167) demonstrate that transpeptidation may complicate the kinetics with these substrates. Their results suggest that hydrolysis or transpeptidation may occur by amine transfer.

With proteins, pepsin shows a broad specificity; e.g. 16 different bonds are split in oxidised insulin (89, 168). Protein substrates remain the basis of most assay methods; Loken *et al.* (169) propose isotopically-labelled serum albumin for such a purpose. Berger, Neumann & Sela (170) make the ingenious suggestion that pepsin can be assayed by its effect on the activity of ribonuclease.

Other "Acid" Proteinases

Rennin.—Rennin was reviewed by Berridge in 1954 (171). It is a rather neglected enzyme, which crystallizes beautifully with a high degree of homogeneity. Jirgensons *et al.* (172) show that impurities in the crystalline en-

zyme may be removed by chromatography on diethylaminoethyl cellulose, and Ernstrom (173) detects electrophoretic inhomogeneity in crystalline preparations. Foltmann (174) describes the partial purification of prorennin.

Other gastric proteinases.—The existence of several pH optima for the digestion of proteins by gastric juice has often been used to infer the presence of enzymes other than pepsin in the juice. Taylor (175, 176) has conducted an extensive study of such systems and finds that the pH-activity relationship depends more on the protein digested than on the gastric preparation used and, more seriously, that the pH-activity curves of pure pepsin or gastric juice are similar against the same protein or synthetic peptide.

On the other hand, Tang *et al.* (177) reported a crystalline enzyme, from human gastric juice, that apparently differs from pepsin. Sedimentation analysis and starch gel electrophoresis indicate relative homogeneity, and the electrophoretic mobility differs from that of human pepsin. Against haemoglobin, an apparent pH optimum of about pH 3 is observed. The specific activity is of the same order as that of pepsin, while the specific milk-clotting activity is less than that of rennin. Further studies on the specificity and amino acid composition of this enzyme may help to establish whether it is related to pepsin. Ryle (178) has isolated two enzymes associated with porcine pepsin by chromatography on diethylaminoethyl cellulose. One of these is almost inactive against haemoglobin but hydrolyzes the pepsin substrate, acetyl phenylalanine diodotyrosine, optimally at pH 1.5 to 2.0. Unlike pepsin, it is stable at pH 6.8. The second enzyme attacks haemoglobin but not the synthetic substrate and is unstable at pH 6.8. The specificity of both enzymes toward the B-chain of insulin resembles, but is not identical with, that of pepsin. It is unfortunate that many of the disagreements about gastric proteinases rest so strongly on kinetic studies with protein substrates, which are capable of such practical and theoretical complexity.

OTHER ENDOPEPTIDASES

Cathepsins

The term "cathepsins," used to describe mammalian intracellular proteinases, tends to imply a homogeneity of properties or function which seems at present unwarranted. Purification of these enzymes is understandably difficult, but the presence of impurities in most cathepsin preparations makes it impossible to define their properties as rigidly as those of most of the enzymes discussed above. It seems possible that a number of cathepsins may, in the future, be classed as "serine," "thiol," "acid," or "metal" proteinases.

Thus, Press & Porter (179), by cellulose ion-exchange chromatography, report the purification, from beef spleen, of a "cathepsin D" which resembles pepsin in its specificity toward the B-chain of oxidised insulin but does not hydrolyse carbobenzoxy glutamyl tyrosine, as does cathepsin A (180). It is not inhibited by "thiol reagents" and has a pH optimum at pH 3 to 4. On the other hand, cathepsin C, further purification of which is reported by De la Haba *et al.* (181), may prove to be a thiol enzyme of the same class as

papain. Fujii & Fruton (182) have now demonstrated that cathepsin C, like papain, is a powerful catalyst for transamidation reactions, whereas cathepsin B is somewhat less effective. The tendency of these enzymes to catalyze such acyl transfer reactions may be related to the steady state concentration of an acyl enzyme intermediate. Where this is high, as in the case of papain with benzoyl arginine ester or amide, the possibility exists of forming a complex with acyl enzyme and an "acyl acceptor." With chymotrypsin and acyl tyrosine amides, however, the rate-limiting step is probably acylation of the serine residue, and Izumiya & Yamashita (183) show that transamidation here is negligible. Further kinetic studies with the cathepsins may help us to understand the mechanism of transamidation.

Proteolytic enzymes are widely distributed in mammalian tissues. Hanson *et al.* (184 to 187) find proteolytic activity in the cytoplasm, nuclei, mitochondria, and microsomal fractions of rat liver and kidney homogenates. They classify the enzymes on the basis of activity toward haemoglobin and some peptides or amino acid esters. Martin & Axelrod (188 to 190) have continued their investigation of proteolytic enzymes in skin. They find some proteinases that are inhibited by DFP, indicating the probable existence of cathepsins of the "serine" proteinase class. Furthermore, some cathepsins are undoubtedly exopeptidases that require specific metal ions for activity. Kenny (191) and Waldschmidt-Leitz & Keller (192) provide recent evidence for such in liver, pancreas or kidney. It is clearly erroneous to assume that the cathepsins are necessarily "thiol" enzymes.

Proteinases from Microorganisms

Collagenase.—Among bacterial proteinases, the collagenase of *Clostridium histolyticum* has been the subject of some interesting research. Purification has been achieved by continuous curtain electrophoresis [Mandl *et al.* (193)], chromatography on diethylaminoethyl cellulose [Grant & Alburn (194)], or starch gel electrophoresis [Gallop *et al.* (195)]; preparations which attack only collagen or gelatin have been reported (193, 195). Seifter *et al.* (196) describe a preparation which is almost homogeneous in the ultracentrifuge and has a molecular weight of 109,000.

Collagenase has an unusually narrow specificity. Michaels *et al.* (197) show that it splits peptide bonds on the N-terminal side of 60 per cent of the glycine residues in collagen. Only glycine residues are found to be N-terminal in the peptides formed, and no specificity is apparent from study of the C-terminal residues of these peptides. The authors show that collagenase is inactive against a wide range of other proteins and peptides and conclude that it is specific for bonds that are N-terminal to —Gly·Pro— sequences in collagen. Their brief report that mixed polymers of proline and glycine are split by collagenase provides support for this hypothesis. Nagai & Noda (198) reached similar conclusions from the action of collagenase on poly (Pro·Leu·Gly), which yields almost entirely Gly·Pro·Leu. They extend the structural requirements to the sequence —Pro·X↓Gly·Pro— since the

peptide Pro·Leu·Gly·Pro·Leu is not hydrolysed by collagenase, whereas Gly·Pro·Leu·Gly·Pro·NH_2 is easily split. Heyns & Legler (199) provide confirmatory evidence for the action of collagenase on collagen and show that Cbz·Pro·Ala¦Gly·Pro·HN_2 is rapidly cleaved, whereas Cbz·Ala·Gly·Pro·NH_2 is split only slowly.

Collagenase is inhibited by chelating agents, thiols, *p*-chloromercuribenzoate, or iodoacetic acid, but not by DFP (196). Seifter *et al.* (196) suggest that it is neither a "thiol" enzyme nor a "serine" enzyme and show that calcium ions are required for adsorption of the enzyme to collagen, while another unknown metal ion also appears to be essential for activity. This enzyme may therefore be a "metal" endopeptidase.

Other proteinases.—Many other proteolytic enzymes from microorganisms have been reported. It is clear that some of these are exopeptidases: for example, an aminopeptidase from *coccus P* [Colobert (200)]. Of particular interest is a proline iminopeptidase, isolated by Sand, Berger & Katchalski (201) from a proline-requiring mutant of *Escherichia coli*, which selectively cleaves N-terminal proline residues from poly L-proline or from salmine. Another unusual enzyme is an extracellular protease, which appears to be of remarkably low molecular weight, purified by Wootton & Hess (202) from the protozoan *Tetrahymena pyroformis W.* Mills & Wilkins (203) report a proteinase from *Proteus vulgaris* that has a molecular weight of 40,000 to 50,000 and is homogeneous on electrophoresis and in the ultracentrifuge. Husain & McDonald (204) have studied an extracellular proteinase from *Micrococcus freudenreichii.* Crystalline proteolytic enzymes from molds are reported by Mizunuma & Iguchi (205), Yoshida & Nagasawa (206), and Nomoto & Narahishi (207). Insects [Brookes & Newburgh (208)] or snake venoms [Henriques *et al.* (209)] are other sources of proteolytic enzymes.

With such a wealth of proteolytic enzymes now at our disposal, may this reviewer plead for agreement on some standard method for comparing their specificity? Many of them may be powerful tools in exploring the structure of proteins, and their action against peptides of known sequence, such as the chains of oxidized insulin, glucagon, or the peptide hormones, would be of great interest. Comparative paper chromatography or electrophoresis of the peptides liberated by different enzymes might simplify such studies.

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TRANSFERASES[1,2]

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Until now the subject of transferases has never been treated in an independent article in the *Annual Review of Biochemistry*. These enzymes have hitherto been dealt with in various chapters, mostly under "Nonoxidative, Nonproteolytic Enzymes." The rapidly increasing number of transferases, as well as the great importance of these enzymes in almost all major metabolic processes, may justify an independent treatment of transferases in these *Reviews*.

The space allotted permits only a selective approach to the subject. With certain exceptions, extensive discussion of the mechanism of transferase actions has been omitted. The mechanism of enzyme action is to be reviewed in another chapter of this volume; furthermore, an up-to-date treatment of mechanisms of transfer enzymes has appeared quite recently (1). Also excluded is the extensive literature on transferring enzymes in protein biosynthesis, also the subject of a separate article.

Definition and Nomenclature

In introducing a new topic, it may be appropriate to discuss briefly questions of definition and nomenclature. In a broader sense, the great majority of all enzymes catalyze transfer reactions. Thus, all hydrolytic actions may be considered transfer reactions, water serving as the acceptor for a part of the substrate molecule. The same applies to the oxidation-reduction enzymes, which transfer hydrogen or electrons or both from a donor to an acceptor. According to common usage, however, neither hydrolyzing enzymes nor oxidoreductases are considered transferases. This term has been reserved for those enzymes which, according to a definition given by the author (2), transfer a part of a donor molecule, except hydrogen or electrons, to an acceptor molecule, and neither the donor nor the acceptor may be water. Although some overlapping between hydrolases and transferases exists and is of considerable theoretical interest, this definition, to our knowledge, has never been seriously contested. On a theoretical basis, there may be good reason to abolish the boundary between transferases and hydrolases; however, this distinction has been kept for practical purposes in all newer text-

[1] The survey of the literature pertaining to this review was completed in September, 1959.

[2] The following abbreviations are used: AMP for adenosine monophosphate; CDP for cytidine diphosphate; CMP for cytidine monophosphate; CTP for cytidine triphosphate; CoA for coenzyme A; GDP for guanosine diphosphate; GMP for guanosine monophosphate; GTP for guanosine triphosphate; IDP for inosine diphosphate; IMP for inosine monophosphate; ITP for inosine triphosphate; P_i for orthophosphate; PP for pyrophosphate; THFA for tetrahydrofolic acid; UDP for uridine diphosphate; UMP for uridine monophosphate; UTP for uridine triphosphate.

books and treatises on enzymes. Accordingly, papers on hydrolyzing enzymes will not be considered here, even if these enzymes show, in addition to their hydrolytic action, a real transfer action according to the definition given above.

Nomenclature of enzymes in general is, at present, in a process of revision. The Enzyme Commission, which was established a few years ago within the framework of the International Union of Biochemistry, intends to devise rules for a rational nomenclature of all enzymes, the main use of which will be for indexing and abstracting purposes. In addition, recommendations for trivial names will be made, as the rational names are often too unwieldy. Although the work of the Commission has not yet been finished, the rules for the rational naming of transferases seem to be quite final and, in part, have already been made public (3). According to these rules, a transferase should be named rationally following the scheme: group donor—group acceptor—group transferred—transferase. Thus, the well-known enzyme catalyzing the reversible transfer of an amino group from L-alanine to α-ketoglutaric acid should rationally be named L-alanine α-ketoglutarate aminotransferase. In regard to the direction to be chosen for enzymes catalyzing reversible reactions, it is suggested that, in all cases where ATP is involved in a transfer reaction, this compound should always be named as the donor, whereas α-ketoglutarate should always be named as the acceptor in amino group transfer. In reactions for which these rules cannot be applied, the substrate with higher mol. wt. should be named as the donor. The latter rule cannot, however, be considered final.

According to these rules, all enzymes catalyzing simple transfer reactions can easily be named rationally. However, with bifunctional or "two-headed" enzymes, which catalyze a group transfer in addition to some other reaction, the system is not yet applicable. It is probable that special rules must be devised for this type of enzyme, the number of which is still small but tends to increase.

In the present review, the rational names will be used in those cases where they are not appreciably longer than the trivial names and are easily understandable. In other cases, only examples will be given in order to make the reader familiar with the new system.

Phosphotransferases

Simple phosphotransferases (kinases).—Work on enzymes catalyzing interconversions between nucleoside mono-, di-, and triphosphates has been very active recently. In calf liver, there are at least two different enzymes transferring a phosphate moiety from nucleoside triphosphates to nucleoside monophosphates. Strominger *et al.* (4, 5) have purified the nucleoside monophosphate kinases from this material and have obtained two fractions, one catalyzing the transphosphorylation from ATP to all ribose mononucleotides but IMP, whereas the other brings about transphosphorylations from any nucleoside triphosphate to AMP. In the course of purification of

the first-named fraction, the ATP-GMP phosphotransferase disappeared completely, and the ATP-AMP phosphotransferase activity was partially removed. This suggests that, on further fractionation, it may be possible to obtain enzyme preparations with even greater specificity. In the case of the fraction catalyzing the transphosphorylation between AMP and the various nucleoside triphosphates, however, there is no indication that further purification may reveal the existence of a whole family of related enzymes. It is interesting to note that, in all fractions, no transphosphorylation has been discovered without participation by adenosine nucleotide. This fact once again illustrates the central role of the adenosine nucleotides in distributing "high energy" phosphate among the nucleotides.

An adenylate kinase has been demonstrated (6) in erythrocytes, with an activity comparable to that in rabbit muscle. Kirkland & Turner (7) describe an enzyme phosphorylating nucleoside diphosphates at the expense of ATP; they obtained and purified this enzyme from pea seeds. Inosine diphosphate and UDP are the preferred substrates, but GDP and CDP are also phosphorylated. The enzyme is widely distributed in plants; it may well be identical with an enzyme from wheat (8) that catalyzes the phosphorylation of UDP to UTP.

Beljanski (9) has found four different enzymes in purified extracts from *Alcaligenes faecalis*, each of them catalyzing the phosphorylation of the four ribose mononucleotides at the expense of ATP. A highly purified enzyme preparation from *Azotobacter vinelandii*, catalyzing the reversible transfer of phosphate from ATP to CMP or deoxycytidine monophosphate, has been described by Maley & Ochoa (10). The reaction products are CDP or deoxycytidine diphosphate, respectively, and ADP.

Various groups of workers have shown that extracts of *Escherichia coli* infected with T2-phage, besides other enzymatic differences, contain phosphotransferases not detectable in extracts from normal *E. coli* cells. Hydroxymethylcytosine deoxynucleotide is not phosphorylated by extracts from normal cells, but, in phage-infected cells, an enzyme or enzyme system that phosphorylates the compound to the di- and triphosphate level is formed within a few minutes after the infection (11, 12). It seems, however, that not only are new phosphotransferases formed after the phage infection, but other enzymes of this action are also changed in their requirements. Thus, Bessman & Van Bibber (13) report that deoxyguanylate kinase from extracts of normal *E. coli* requires K^+ in addition to ATP and Mg^{++} for its activity, whereas K^+ is not necessary for the activity of the enzyme in extracts from the phage-infected organism.

1,5-Anhydro-D-glucitol-6-phosphate is a very effective inhibitor of animal hexokinases (14), whereas its effect on enzymes catalyzing reactions of glucose-6-phosphate is negligible. Thus, glucose oxidation in heart muscle homogenates is completely suppressed by the compound, whereas the oxidation of glucose-6-phosphate is not impaired. The compound may become a useful tool in the study of metabolic pathways. Sols *et al.* (15) reinvestigated

the substrate specificity of bakers' yeast hexokinase. The results indicate that the general pattern of substrate specificity of this enzyme follows the general line of that of brain hexokinase [Sols & Crane (16)]. In the writer's laboratory, however, it was found that an impure hexokinase preparation from yeast is able to phosphorylate *myo*-inositol (17), and later experiments with a highly purified yeast hexokinase preparation showed that this activity, although weak, is, in fact, caused by the enzyme. This is in contrast to brain hexokinase which does not phosphorylate inositol (16).

In *Lactobacillus plantarum*, 2-deoxyribose induces the formation of an enzyme phosphorylating this compound, as well as ribose, at the expense of ATP. Ginsburg (18) suggests the name deoxyribose kinase for the enzyme, because it differs from the ribokinase which can be induced in *L. plantarum* but does not phosphorylate deoxyribose. The dual specificity of deoxyribose kinase toward ribose and deoxyribose is similar to that observed with an enzyme from calf liver (19).

Enzymes capable of transferring phosphate residues from ATP to D-glucuronic acid, D-galactose, and L-arabinose, forming the corresponding sugar-1-phosphates, exist in mung beans as well as in other plants (20, 21).

The so-called threonine synthase, an enzyme system which catalyzes the formation of threonine from homoserine (22, 23), consists of two components, one of which phosphorylates homoserine, whereas the other transforms homoserine phosphate to threonine and inorganic phosphate. Wormser & Pardee (24) investigated the enzyme catalyzing the first step of the reaction sequence, the homoserine kinase, from broken-cell preparations of *E. coli* and from yeast. It was found that threonine competitively inhibits the enzyme action, thus preventing the formation of its own precursor. It appears that, at least in *E. coli*, this feedback mechanism plays a role in controlling the rate of biosynthesis of threonine. Although the system in yeast has been studied in less detail, the data seem to indicate the existence of a similar mechanism.

An enzyme from rat liver mitochondria which phosphorylates D-glycerate to 2-phospho-D-glycerate in the presence of ATP and Mg^{++} has been studied by Lamprecht *et al.* (25). This enzyme seems to be identical with an enzyme described by previous workers (26, 27); however, Lamprecht and associates (25) report evidence that the reaction product of this enzyme is 2-phosphoglycerate rather than 3-phosphoglycerate as the results of the earlier workers would imply. To distinguish between the two isomeric phosphoglycerates, complex enzymatic tests had to be developed, as all previously proposed methods were not sufficiently specific.

Various aspects of the action of pyruvate kinase (rational name: ATP-pyruvate phosphotransferase) have been thoroughly investigated. In addition to ADP, GDP and IDP can also act as phosphate acceptors (28); the observed reaction of CDP and UDP is considered, by the author, to be caused by a contamination of the enzyme preparation. On the other hand,

Leloir and co-workers (29) use this enzyme successfully as an analytical tool for the determination of UDP.

The so-called "fluorokinase," an enzyme catalyzing the CO_2-dependent phosphorylation of fluoride at the expense of ATP, has been isolated in crystalline form from rabbit muscle and is identical with pyruvate kinase (30). The mechanism of the CO_2-dependent fluorophosphate formation catalyzed by the enzyme is, however, still unknown.

The very important problem of whether the reversal of pyruvate kinase action, i.e., the formation of phosphoenolpyruvic acid and ADP from ATP and pyruvic acid, plays any role in the conversion of pyruvic acid to glycogen has also been studied. Krimsky (31) describes enzyme systems in which the high ratio of ATP to ADP maintained by rephosphorylation of ADP permits phosphoenolpyruvate synthesis. This author and McQuate & Utter (32) report values for the equilibrium constant of the reaction which are in relatively good agreement. The apparent equilibrium constant lies even farther toward pyruvate than had been suggested by the earlier measurements of Meyerhof & Oesper (33). Kinetic studies, however, seem to indicate that the ratio between the maximal forward and backward reaction rates is not markedly unfavorable for phosphoenolpyruvate synthesis. In both papers (31, 32), it is pointed out that the data obtained do not rule out glycogen synthesis by the way of the pyruvate kinase reaction. Any further attempt to assess the importance of this reaction for glycogen synthesis in a particular tissue, however, will require a knowledge of the amounts and properties of this enzyme in the tissue as well as information on the *in vivo* level of metabolites. Present-day knowledge on creatine kinase, arginine kinase, and similar enzymes has been very competently reviewed by Ennor & Morrison (34).

The only pathway for the formation of phosphatidic acids hitherto known has been the synthesis from α-glycero-phosphate and CoA thiol esters of fatty acids, as described by Kornberg & Pricer (35). Hokin & Hokin (36) now present convincing evidence for the existence, in brain, of an enzyme which catalyzes the direct phosphorylation of diglyceride to phosphatidic acid, according to the equation

$$\text{ATP} + \text{diglyceride} \rightarrow \text{phosphatidic acid} + \text{ADP}.$$

The enzyme was found in soluble extracts from deoxycholate-treated brain microsomes; it seems to be also present in the intact microsomes, but added substrate is not utilized unless surface-active agents like deoxycholate are added. The nature of the fatty acyl residues of the diglyceride substrate is important; the substrate most easily phosphorylated is diglyceride from cabbage phosphatidic acid, whereas diglyceride from brain lecithin and 1-palmityl-2-oleyl diglyceride are much less effective.

In recent times, the study of the biosynthetic pathways leading to the terpenes and steroids has been very active. Cell-free yeast extracts are able to

synthesize squalene from mevalonic acid in the presence of ATP, triphosphopyridine nucleotide (reduced form), and Mg^{++} (37). The first step in this reaction sequence is the phosphorylation of mevalonic acid. Tchen (38) describes a partial purification of the mevalonic kinase from yeast. This enzyme catalyzes the formation of 5-phosphomevalonic acid and ADP from ATP and mevalonic acid. The lack of specificity with regard to the donor nucleotide is somewhat remarkable: ATP can be replaced by CTP, GTP, or UTP; ADP, however, is inactive as a donor. In all other respects, the enzyme has the properties of most other kinases. Mevalonic kinase from pig liver has also been purified (39).

The next step in the pathway to squalene is also catalyzed by a phosphotransferase. Chaykin and co-workers (37) had already found that a yeast enzyme fraction which converted mevalonic acid to isopentenylpyrophosphate, a further intermediate in the reaction sequence, produced in addition to 5-phosphomevalonic acid and isopentenylpyrophosphate a third compound to which the structure of 5-pyrophosphorylmevalonic acid was tentatively assigned. These observations were substantiated by Henning *et al.* (40), who report a partial purification of the enzyme responsible for the formation of 5-pyrophosphorylmevalonic acid. This enzyme catalyzes the reaction

ATP + 5-phosphomevalonic acid → 5-pyrophosphorylmevalonic acid + ADP

[cf. also Bloch, Chaykin & Phillips (41)]. Henning and co-workers (40) call this enzyme phosphomevalonic kinase; the rational name is to be ATP-phosphomevalonate kinase.

Marcus & Elliott (42) have found that acetokinase (ATP-acetate phosphotransferase) of *E. coli* phosphorylates fluoroacetate almost as readily as acetate.

Camiener & Brown (43), who studied the biosynthesis of thiamine and thiamine pyrophosphate in cell-free yeast extracts, found that various phosphorylation steps are involved in this process. The authors report that the primary steps in thiamine synthesis from the pyrimidine and thiazole moieties are catalyzed by phosphotransferases bringing about the reactions

ATP + 2-methyl-4-amino-5-hydroxymethylpyrimidine
→ 2-methyl-4-amino-5-hydroxymethylpyrimidyl pyrophosphate + AMP

and

ATP + 4-methyl-5-(β-hydroxyethyl)-thiazole
→ 4-methyl-5-(β-hydroxyethyl)-thiazole phosphate + ADP.

In the case of the first reaction, it has not yet been established whether the pyrophosphorylation occurs in one step, yielding AMP as a product, or in two steps, in which case two molecules of ATP would be required and two molecules of ADP would result.

Although it has been assumed that "high energy phosphate" transfer is obligatorily mediated by ADP or other diphosphonucleosides, Cori *et al.*

(44) present strong evidence for the existence of enzymes catalyzing such transphosphorylations without the participation of nucleoside diphosphates. An enzyme that transfers the carboxyl phosphate of 1,3-diphosphoglycerate to creatine without mediation by ADP has been obtained from rat muscle. The possibility of ADP as a contaminant in the preparation was excluded by enzymatic tests capable of detecting the smallest amounts of ADP. The enzyme described is different from creatine kinase in various respects, e.g., its heat stability and the inhibition of its action by ammonium sulfate. Furthermore, heart muscle contains creatine kinase, but not the direct-way phosphotransferase. The authors suggest the name 1,3-diphosphoglycerate-creatine phosphotransferase for the new enzyme; this name is consistent with the rational nomenclature outlined above.

Enzymes and enzyme systems catalyzing more complex reactions involving phosphoryl transfer.—Various complex biosynthetic reactions seem to depend on a phosphoryl transfer, although the phosphorylated intermediates to be postulated have, in most cases, not yet been identified. In some of these cases, further purification may reveal the existence of more than one enzyme; in other cases, however, it seems that the reactions are actually catalyzed by single enzyme entities, and the intermediates are probably either very unstable or, for the short time of their existence, tightly bound to the enzyme protein.

In the enzymatic reaction system leading to the synthesis of inosinic acid, the formation of N-(5-amino-1-ribosyl-4-imidazolyl-carbonyl)-L-aspartic acid from 5-amino-1-ribosyl-4-imidazole-carboxylic acid-5′-phosphate and L-aspartic acid proceeds as a transfer reaction and requires the presence of ATP. Among the reaction products, ADP and orthophosphate are to be found in stoichiometric amounts, which suggests the intermediate formation of a phosphorylated product (45).

The synthesis of adenylosuccinate, the precursor of AMP, from IMP and aspartic acid, also seems to proceed via a phosphorylated intermediate. This reaction has been studied in two different laboratories, and the results differ to a certain extent. Davey (46) claims that an enzyme preparation from mammalian muscle requires the presence of GTP in the reaction mixture, whereas, according to Yefimochkina & Braunstein (47), who worked with a similar system, ATP is necessary and cannot be substituted by GTP.

The decarboxylation of 5-pyrophosphorylmevalonic acid to isopentenylpyrophosphate also requires ATP; the enzyme responsible for this action has been studied in some detail by De Waard, Phillips, & Bloch (48). With a purified enzyme preparation, it was found that all products of the reaction

$$\text{ATP} + \text{5-pyrophosphorylmevalonic acid} \rightarrow \text{isopentenylpyrophosphate} + CO_2 + \text{ADP} + P_i$$

are formed in stoichiometric amounts. Using ^{32}P-labeled ATP, no incorporation of label into isopentenylpyrophosphate took place, and all the activity was found in the orthophosphate. The authors suggest a mechanism in-

volving the intermediate formation of an unstable or enzyme-bound product, which they visualize as 5-pyrophosphorylmevalonic acid phosphorylated at the 3-hydroxyl group, which finally breaks down to isopentenylpyrophosphate, CO_2, and orthophosphate.

A 500-fold purification of the propionyl-CoA carboxylation enzyme from pig heart has been achieved by Tietz & Ochoa (49). According to the authors, the reversible reaction

$$\text{ATP} + \text{CO}_2 + \text{propionyl-CoA} \rightleftharpoons \text{ADP} + \text{P}_i + \text{methylmalonyl-CoA}$$

is probably caused by a single enzyme entity. In the absence of propionyl-CoA, no "activation" of CO_2 seems to occur. The purified enzyme does not contain biotin or any dissociable factor.

Carbamylphosphate synthetase action also seems to involve a phosphotransfer reaction. Metzenberg and associates (50, 51), who have studied an enzyme preparation from mammalian liver that catalyzes the formation of carbamylphosphate from ATP, CO_2, and NH_3, postulate the intermediate formation of an activated form of CO_2, which then reacts with ammonia. The first step is envisaged as:

$$\text{ATP} + \text{CO}_2 \rightarrow \text{"active" CO}_2 + \text{ADP} + \text{P}_i$$

and is apparently irreversible. The presence of acetylglutamic acid is required for the overall process; the role of this compound is not yet clear. It was found that neither the oxygen of water nor the oxygen of the terminal phosphate of ATP is incorporated into acetylglutamic acid during the synthetase reaction, which implies that no compound in which carboxyl or acetyl oxygen of acetylglutamate is replaced by another element can act as an intermediate.

The "activation" of formic acid is another important process involving a phosphotransfer reaction. Whiteley, Osborn & Huennekens (52) have purified an enzyme from *Micrococcus aerogenes* that catalyzes the reaction

$$\text{ATP} + \text{formate} + \text{THFA} \rightleftharpoons \text{10-formyl-THFA} + \text{ADP} + \text{P}_i$$

and have studied the specificity and stoichiometry of the reaction. The formation of a phosphorylated intermediate, probably a derivative of tetrahydrofolic acid, had been previously postulated (53). Whiteley *et al.* now report that a synthetically obtained phosphorylated derivative of THFA is active in the overall process (54), but the nature of the intermediate product formed in the enzymatic process is not yet clear.

Rabinowitz (55) has studied the enzymatic conversion of formiminoglycine, ADP and P_i to ATP, glycine, and formic acid in extracts of *Clostridium* sp. This process, which obviously also comprises a phosphotransfer reaction, requires the presence of a heat-stable, but oxygen-labile, factor in the boiled extracts of the organism, which can be replaced by synthetic *d,l*-THFA. The

factor present in the extracts is, however, not identical with THFA, but seems to be a higher glutamic acid conjugate.

As already pointed out by various authors [cf. e.g., Simkin (56)], the formation of amino acid adenylates does not represent the only mechanism by which amino acids can be "activated." Cormier *et al.* (57, 58) have purified a glycine-"activating" enzyme from *Photobacterium fischeri*, the action of which seems to follow the equation

$$\text{enzyme} + \text{ATP} + \text{glycine} \rightarrow \text{enzyme-glycyl phosphate} + \text{ADP}.$$

This phosphotransfer reaction shows a close analogy to the mechanism postulated for the glutamine synthetase reaction (59, 60); "active glycine" may play a role in the biosynthesis of glycinamide ribotide.

Phosphomutases.—The so-called phosphomutases must be considered a special type of phosphotransferase, catalyzing seemingly intramolecular transfers of phosphate residues. Glaser *et al.* (61) describe a partially purified yeast enzyme which brings about specifically the conversion of α-mannose-1-phosphate to mannose-6-phosphate. The enzyme is not identical with glucose phosphomutase; it is inactive in the absence of either α-glucose-1,6-diphosphate or α-mannose-1,6-diphosphate, which both act equally well as coenzymes.

Ito & Grisolia (62) have purified a phosphoglycerate mutase from wheat germ and have found that the enzyme does not require 2,3-diphosphoglycerate for its action. This is in contrast to the known enzymes of this specificity from mammalian sources and from yeast, which all require the presence of 2,3-diphosphoglycerate as coenzyme. Accordingly, the mechanism of action of the wheat germ enzyme must be different from that of the hitherto known phosphoglycerate mutases. A comparative study of phosphoglycerate mutases from 26 different biological sources revealed that only enzymes from seeds are capable of catalyzing the phosphomutase reaction in absence of the coenzyme (63). A study of the donor and acceptor specificity of crystalline phosphoglycerate mutase from muscle has been made by Pizer & Ballou (64). If the compound is to act as an acceptor, a negatively charged group is required on carbon 1, carbons 2 and 3 must carry hydroxyls, which could take up a D-*erythro* configuration, and one of these hydroxyls must be esterified with phosphate. A donor has to be phosphorylated at both hydroxyl positions.

Diphosphoglycerate mutase, the enzyme catalyzing the conversion of 1,3-diphosphoglycerate to 2,3-diphosphoglycerate, has been purified from chicken breast muscle (65). As already pointed out by Rapoport & Luebering (66, 67), the reaction seems to be irreversible.

Enzymes Catalyzing the Transfer of Substituted Phosphoryl Residues

Enzymes that transfer substituted phosphoryl residues, i.e., nucleotidyl, phosphoribosyl, and cholinephosphoryl residues, will be discussed here.

Most, but not all, of these enzymes have hitherto been classified as pyrophosphorylases. A classification according to the group transferred is, however, more consistent with the general classification principles for transferases.

Enzymes of nucleic acid synthesis.—In a study of the action of the polynucleotide phosphorylase (rational name: nucleoside diphosphate polynucleotide nucleotidyl transferase) of *Azotobacter vinelandii*, Ortiz & Ochoa (68), when incubating the enzyme with mixtures of ^{32}P-labeled ADP or UDP with the other three unlabeled ribonucleoside diphosphates, obtained RNA-containing radioactive phosphate. After hydrolysis of the RNA thus synthesized with phosphodiesterase from snake venom, nucleoside-5′-phosphates were isolated; of these only AMP and UMP were labeled. Hydrolysis by spleen phosphodiesterase or alkali, however, yielded nucleoside-3′-phosphates (or an isomeric mixture of 3′- and 2′-monophosphates), all of which were labeled approximately to the same extent. This shows that, by the action of the polynucleotide phosphorylase, the labeled nucleotides are randomly distributed throughout the polynucleotide chain.

Škoda and associates (69) investigated the effects of 6-azauridine diphosphate on the polynucleotide phosphorylase of *E. coli*. It has been found that this antagonist does not only inhibit the incorporation of pyrimidine nucleotides, but also that of purine nucleotides, into the polynucleotide chain. The effect is quite specific; 6-azuridine monophosphate does not show any inhibitory action.

Hakim (70) presents evidence for the existence of a polynucleotide phosphorylase in human urine and sperm. From a reaction mixture containing only a single nucleoside diphosphate, homogeneous polymers are obtained, while from mixtures of the four different ribonucleoside diphosphates, heterogeneous polymers are formed. The enzyme has been partially purified. This is the first report on the existence of this type of enzyme in mammalian material; all enzymes of this specificity hitherto described had been discovered in microorganisms.

Most existing evidence, however, seems to show that the main precursors for RNA synthesis in mammalian material are the nucleoside triphosphates rather than the diphosphates. This seems to be convincingly established for the attachment of nucleotides as terminal residues of the polynucleotide chain [cf. e.g., Hecht *et al.* (71)], but two recent reports indicate that similar mechanisms prevail also for the incorporation into the inner nucleotide positions of RNA [Edmonds & Abrams (72); Weiss & Gladstone (73)]. The latter authors, working with rat liver nuclei, observed an incorporation of cytidylate from CTP which was ^{32}P-labeled at the innermost phosphorus atom. After hydrolysis of the RNA thus formed, label was found in all the mononucleotides; this can be considered proof of RNA incorporation into the interpolynucleotide linkages rather than into the terminal positions. The incorporation of CTP is markedly enhanced by ATP, UTP, and GTP, the corresponding nucleoside diphosphates being much less effective. All evidence

indicates that the reaction mechanism in this system resembles that described for DNA formation by DNA polymerase of *E. coli* rather than that for RNA synthesis by polynucleotide phosphorylase.

The fact that the composition of DNA synthesized by the action of DNA polymerase (rational name: deoxynucleoside triphosphate DNA deoxynucleotidyl transferase) reflects completely the composition of the primer-DNA present in the reaction mixture strongly suggests that a template mechanism exists in addition to simple transfer (74). Kornberg *et al.* (11) also describe DNA synthesis in T2-phage infected *E. coli*. In extracts of infected cells, when deoxyadenosine triphosphate, deoxyguanosine triphosphate, thymidine triphosphate, and deoxycytidine triphosphate are present, DNA synthesis is absent or at least markedly depressed, whereas, in extracts from normal cells under the same conditions, vigorous DNA synthesis takes place. When, however, in the extracts from infected cells, deoxycytidine triphosphate is replaced by hydroxymethylcytosine deoxynucleoside triphosphate, a five- to tenfold increase in DNA synthesis over the level in the extracts from uninfected cells is observed.

Bollum (75) has studied a DNA polymerase in a soluble fraction from calf thymus. The system incorporates tritium-labeled thymidine triphosphate into DNA; it has an absolute requirement for DNA (as primer) and Mg^{++} and exhibits maximal activity in the presence of deoxycytidine triphosphate, deoxyadenosine triphosphate, and deoxyguanosine triphosphate.

Hurwitz (76) describes an enzyme system from *E. coli* which incorporates ribonucleotides, furnished as the triphosphates, into polynucleotides with the properties of DNA. It seems that the incorporation of ribonucleotides is favored when the corresponding deoxynucleotides are lacking. Degradation experiments with the products of this reaction indicate the formation of a 3′,5′-diester linkage between ribo- and deoxynucleotide residues. The biological significance of this reaction cannot yet be assessed.

Adenylyl transferases.—Probably the most important case of enzymatic adenylyl transfer encountered thus far is the formation of the so-called active amino acids according to the general equation

$$\text{ATP} + \text{amino acid} + \text{enzyme} \rightleftharpoons \text{enzyme-amino acyl adenylate} + \text{PP}.$$

As protein biosynthesis is, however, the subject of another review, only those reactions of this type probably not involved in protein biosynthesis will be treated here.

The biosynthesis of carnosine and anserine has been studied with enzyme preparations from muscle (77 to 80). Carnosine synthetase catalyzes the formation of carnosine or anserine from β-alanine and L-histidine or 1-methyl-L-histidine, respectively, in the presence of ATP and Mg^{++}. Synthetic β-alanyl adenylate can replace β-alanine, ATP, and Mg^{++}. This seems to imply that the primary step in carnosine and anserine synthesis is the transfer of an adenylyl residue from ATP to β-alanine (78); the resulting β-alanyl adenylate seems to be bound to the enzyme protein. In a second step, the β-alanyl

moiety is transferred from the enzyme-β-alanyl-adenylate complex to histidine or methylhistidine. No carnosine formation was observed when β-alanyl phosphate was used in place of β-alanyl adenylate. The latter compound is extremely reactive; even in absence of the enzyme, some carnosine is formed from synthetic β-alanyl adenylate and histidine. Similar observations with amino acyl adenylates have been made by other authors (81, 82).

A specific enzyme catalyzing the "activation" of D-alanine has been found by Baddiley & Neuhaus (83) in extracts of *Lactobacillus arabinosus*, which contains D-alanyl residues within the molecules of teichoic acid. The "activation" reaction is completely analogous to that observed with the "natural" amino acids of the L-series:

$$\text{ATP} + \text{D-alanine} + \text{enzyme} \rightarrow \text{enzyme-D-alanyl-adenylate} + \text{PP}.$$

The full description of Petrack & Ratner's (84) work on the biosynthesis of argininosuccinate from citrulline and aspartic acid in the presence of ATP has appeared. Further work from the same laboratory (85) indicates that citrulline adenylate, either free or enzyme-bound, acts as an intermediate in the process. Thus, the first step of argininosuccinate formation may be thought of as an adenylyl transfer from ATP to the ureido oxygen of citrulline, whereas, in the second step, the citrullyl residue is transferred from citrullyl adenylate to aspartic acid.

An enzyme catalyzing the formation of "active" ammonia, i.e., the phosphoryl amide derivative of AMP, has been discovered in *Mycobacterium avium* by Katunuma (86). It catalyzes the reaction

$$\text{ATP} + \text{NH}_3 \rightarrow \text{adenylyl amidate} + \text{PP}.$$

It seems that enzymes of this specificity occur only in bacteria and yeast and could not be found in higher animals or plants (87). "Active" ammonia serves as an amino group donator for the formation of L-alanine from pyruvate and of L-glutamic acid from α-ketoglutarate (88). It may be that the enzyme plays a role in the biosynthesis of amino acids in lower organisms.

ATP-sulfurylase (rational name: ATP sulfate adenylyl transferase) has been purified from yeast by Wilson & Bandurski (89). Selenate can also act as adenylyl acceptor, whereas, in the presence of sulfite, chromate, tungstate, or molybdate, free AMP and pyrophosphate are formed. The latter reaction bears some resemblance to the well-known arsenolysis of phosphate esters.

Uridylyl transferases.—It is now well established that sugar uridine diphosphates are the precursors of many oligosaccharides, polysaccharides, and glycosides. The formation of these important compounds is a uridylyl transfer and follows the general equation

$$\text{UTP} + \text{sugar-1-phosphate} \rightleftharpoons \text{UDP-sugar} + \text{PP}.$$

Various new reports extend our knowledge of this reaction type. Hassid *et al.*

(21, 90) have studied the enzymatic formation of UDP-sugars in mung beans. UDP-D-glucose, UDP-D-galactose, UDP-D-xylose, UDP-L-arabinose, UDP-D-glucuronic acid, UDP-D-galacturonic acid, and UDP-acetyl-D-glucosamine are formed in the plant extracts by the mechanism described above; there is not yet sufficient evidence available to estimate the number of enzymes responsible for these different reactions. Strominger & Smith (91) report on the purification and properties of enzymes, from calf liver and *Staphylococcus aureus*, catalyzing the synthesis of UDP-acetyl-D-glucosamine from UTP and acetyl-D-glucosamine-1-phosphate and also the reverse reaction, the pyrophosphorolytic cleavage of the sugar nucleotide.

Reid (92) has found that UDP-glucose formation in homogenates from rat liver cells is almost entirely associated with the supernatant fraction. This is in apparent contrast to the findings of Smith & Mills (93), who could not observe a pyrophosphorolysis of UDP-glucose in the supernatant. Reid (92) points out that possibly the enzyme in the supernatant can synthesize only UDP-glucose from UTP and glucose-1-phosphate and is not capable of catalyzing the reverse reaction, the pyrophosphorolytic breakdown of the sugar nucleotide. A similar situation was found previously in the case of UDP-acetyl-D-glucosamine synthesis from UTP and acetyl-D-glucosamine-1-phosphate (94).

The existence of different enzymes for the synthesis of UDP-glucose from UTP and glucose-1-phosphate and of UDP-acetylglucosamine from UTP and acetylglucosamine-1-phosphate as well as for the transfer of a uridylyl residue from UDP-glucose to galactose-1-phosphate has been established in extracts of a non-capsulating strain of *Pneumococcus* [Smith *et al.* (95)].

Cytidylyl- and cholinephosphoryl transferases.—Kennedy and co-workers (96) have continued their work on enzymes catalyzing the biosynthesis of lecithin and phosphatidylethanolamine. The discovery of deoxycytidine diphosphate-choline and -ethanolamine in diverse biological materials has made it desirable to study the activity of these substances in the enzyme systems responsible for lecithin and phosphatidylethanolamine biosynthesis. The enzymes transferring cytidylyl residues from CTP to choline and ethanolamine are capable of forming deoxycytidine diphosphate-choline and -ethanolamine from deoxycytidine triphosphate, at about the same rate as the ribonucleotide derivatives from CTP. Of the coenzymes transferring cholinephosphoryl and ethanolaminephosphoryl residues to α,β-diglyceride, deoxycytidine diphosphate-choline is almost as good a donor as CDP-choline, whereas deoxycytidine diphosphate-ethanolamine is much less active in the system than CDP-ethanolamine.

The ultimate step in the biosynthesis of sphingomyelin has been elucidated by Sribney & Kennedy (97). In chicken liver and in mammalian tissues, it is possible to demonstrate an enzyme that catalyzes the transfer of the cholinephosphoryl moiety of CDP-choline to the free primary hydroxyl group of the sphingosyl part of ceramide, according to the equation

CDP-choline + ceramide → sphingomyelin + CMP.

In analogy to the enzyme participating in lecithin synthesis, which had been named PC-glyceride transferase, the authors suggest the name PC-ceramide transferase for the new enzyme; the rational name would be CDP-choline ceramide cholinephosphoryl transferase. The enzyme is highly specific both for CDP-choline and ceramide. The sphingosine moiety of the ceramide must have the *trans* configuration at the double bond, and the hydroxyl group on carbon 3 must have the *threo* relationship to the amino group on carbon 2. This is somewhat surprising since the sphingosine moiety in sphingomyelin isolated from tissues is entirely, or at least predominantly, in the *erythro* configuration.

Agranoff *et al.* (98) have studied inositol phosphatide biosynthesis in the insoluble residue of guinea pig mitochondria. This system catalyzes an incorporation of inositol in the presence of CDP-choline, phosphatidic acid, and Mg^{++}. On the basis of their experimental findings, the authors suggest that the primary reaction in the sequence leading to the formation of inositol phosphatides is the formation of CDP-D-α,β-diglyceride from CDP-choline and phosphatidic acid. This is followed by a second reaction step in which the phosphatidic acid residue is transferred from CDP-D-α,β-diglyceride to a hydroxyl group of free inositol. The suggested pathway is considerably different from the known pathways for the biosynthesis of other phospholipids, e.g., lecithin and phosphatidylethanolamine, insofar as inositol, in contrast to choline and ethanolamine, appears to be incorporated into the lipid without being previously phosphorylated.

The mechanism for the biosynthesis of inositol monophosphatide, as proposed by Agranoff and co-workers (98), has not remained uncontested. Paulus & Kennedy (99), working with homogenates of guinea pig liver, find a specific requirement for CTP in the biosynthesis of inositol monophosphatide, whereas CDP-choline is not active. Thompson *et al.* (100) also present evidence that the mechanism of inositol phosphatide biosynthesis is more complex than that proposed by Agranoff and associates (98). Obviously, further work will be necessary for the complete elucidation of the transfer reactions involved in this process.

Enzymes and enzyme systems catalyzing more complex reactions involving the transfer of substituted phosphoryl residues.—Adenylyl transfer seems to be involved in GMP biosynthesis. In continuation of earlier work, Abrams & Bentley (101) have purified from calf thymus the enzyme catalyzing the amination of xanthosine monophosphate by L-glutamine. An investigation of the reaction mechanism strongly suggests that the overall equation for this amination process is:

ATP + xanthosine monophosphate + glutamine + H_2O
→ GMP + glutamic acid + AMP + PP.

It was found that the enzyme did not catalyze an exchange between ATP

and ^{32}P-labeled pyrophosphate; a reaction of the pyrophosphorylase type is therefore improbable. On the other hand, ^{18}O is transferred from xanthosine monophosphate to AMP, which indicates some sort of intermediate bonding of the oxygen of xanthosine monophosphate to the phosphorus of AMP. High concentrations of ammonium salts may replace glutamine; this is in agreement with the observations of Moyed & Magasanik on an enzyme of similar action from *Aerobacter aerogenes* (102).

Brown (103) has shown that extracts from bacterial and mammalian sources contain an enzyme which converts 4′-phosphopantothenic acid and cysteine to 4′-phosphopantothenyl-cysteine. For the reaction in bacterial extracts, CTP was specifically required. The function of CTP in this process may either be an "activation" of the carboxyl group of 4′-phosphopantothenic acid in a manner similar to ATP in the "activation" of pantoic acid for the synthesis of pantothenic acid or a reaction of CTP with 4′-phosphopantothenic acid to give CDP-pantothenic acid, which might serve as the true substrate for the coupling reaction. The mammalian enzyme differs from the bacterial enzyme insofar as it can use, as well as CTP, any of the other nucleoside triphosphates.

Some interesting aspects of the enzymatic formation of phosphoribosylamine from phosphoribosyl pyrophosphate, which involves a phosphoribosyl transfer, have been studied by Wyngaarden & Ashton (104, 105). It has been found that the reaction

$$\alpha\text{-phosphoribosylpyrophosphate} + \text{glutamine} + H_2O \rightarrow \beta\text{-phosphoribosylamine} + \text{glutamic acid} + PP$$

seems to be a likely site for the operation of a feedback control of the synthesis of purine nucleotides. IMP, AMP, ADP, ATP, GMP, and GDP inhibit the action of the enzyme, whereas deoxyadenosine monophosphate, deoxyguanosine monophosphate, 2′,3′-AMP, IDP, ITP, GTP, ribose-5-phosphate, purine bases and nucleosides, pyrimidine nucleotides, and pyrophosphate are without influence.

Glycosyl Transferases

Among the enzymatic reactions involving glycosyl transfer, it is possible to distinguish three types of reactions: (*a*) glycosyl transfer in which sugar nucleotides participate; (*b*) glycosyl transfer with sugar phosphates as participants, e.g., the reaction catalyzed by phosphorylase; and (*c*) glycosyl transfer in which no phosphorylated sugar compound is involved, e.g., the reaction catalyzed by dextransucrase.

Glycosyl transferases with sugar nucleotides as substrates.—Until quite recently it was generally accepted that α-glucans like starch and glycogen are synthesized by the action of phosphorylase, although, according to the known equilibrium conditions of the phosphorylase reaction, the ratio of orthophosphate to glucose-1-phosphate would have to be very low to make a net synthesis of the polysaccharide possible. Various reports indicate, how-

ever, that the conditions in living tissue rarely, if ever, favor this direction of the reaction. Furthermore, it has been shown that the activation of glycogenolysis by various factors, such as epinephrine, glucagon, and high Na^+ concentration, can be attributed to increased phosphorylase activity. Thus, it seemed well established that the major importance of phosphorylase lies in α-glucan catabolism rather than in anabolism. The reluctance to abandon the concept that phosphorylase is responsible for glycogen and starch biosynthesis has certainly been caused by the fact that, until quite recently, no enzyme mechanism to which these processes could be attributed has been known. The central role of UDP-glycosides as glycosyl donors in the formation of various glycosides, oligosaccharides, and polysaccharides has induced Leloir and co-workers, who had already played a major role in the elucidation of these mechanisms, to search for enzymes synthesizing α-glucans by a similar pathway. Leloir & Cardini (106) reported, in 1957, on a liver enzyme capable of catalyzing a reaction which may be formulated as:

$$\text{UDP-glucose} + \text{primer} \rightarrow \text{glucosyl-}\alpha(1 \rightarrow 4)\text{-primer} + \text{UDP}.$$

The rational name of such an enzyme should be: UDP-glucose α-glucan $\alpha(1\rightarrow4)$-glucosyl transferase.

In continuation of this work, Leloir and associates (29) describe the purification of an enzyme of the same action from skeletal muscle. A study of the primer requirement revealed that the best primer is glycogen, and its activity is decreased by degradation. The primer requirements of the new enzyme somewhat resemble those of muscle phosphorylase. However, glucose-6-phosphate has a strong activating effect on the enzyme; the mechanism of this effect is not yet clear. When incubating ^{14}C-labeled UDP-glucose with enzyme and primer, radioactive glycogen is formed; if this product is treated with β-amylase, all radiocarbon is split off. This indicates that the glycogen-forming enzyme attaches the glucose residues to the primer by $\alpha(1\rightarrow4)$-linkages. The enzyme is to be found mainly in skeletal muscle, heart muscle, and liver.

Several reports of work on the same enzyme confirm the Argentine results and support the assumption that the uridine-linked pathway is mainly responsible for glycogen synthesis in animal tissues [Hauk & Brown (107); Robbins, Traut & Lipmann (108); Villar-Palasi & Larner (109)]. Various nucleotides and, also, phlorizin, a typical phosphorylase inhibitor, inhibit the glucosyl transfer from UDP-glucose to glycogen. Villar-Palasi (110) announces the 100-fold purification of the UDP-glucose α-glucan $\alpha(1\rightarrow4)$-glucosyl transferase.

Burton, Sodd & Brady (111) have investigated the biosynthesis of neutral glycolipids in the microsomal fraction from young rats. They report on an enzyme catalyzing a galactosyl transfer from UDP-galactose to a lipid acceptor endogenous to the microsomal fraction. The product formed by this reaction is a cerebroside, probably N-cerebronyl-O^1-galactosyl sphingosine.

Various reports on UDP-glycosides as glycosyl donors for the formation

of several plant products emphasize the importance of this mechanism. Glucosyl residues are transferred from UDP-glucose to various acceptors to form β-glycosidic bonds in products like gentiobiose, arbutin, salicin, and various other phenyl-β-glycosides. Such transfer reactions have been investigated in wheat germ (112, 113) and in *Vicia faba* (114). Since the D-glucosyl residue in UDP-glucose is α-glucosidically bound, the formation of β-glycosidic bonds by the transfer reaction must proceed with inversion of the configuration. Although several analogous cases are known, the mechanism of inversion at C-1 of the glucose moiety during the transfer reaction has not yet been elucidated.

From asparagus shoots, an enzyme has been prepared which is capable of catalyzing the transfer of D-xylose residues from UDP-D-xylose to $\beta(1\rightarrow4)$-linked xylooligosaccharides of a polymerization degree from 2 to 5 (115). This seems to be the first case of an enzyme catalyzing a pentosyl transfer; the biological implications of the reaction are not yet clear.

Glycosyl transferases with sugar phosphates as substrates.—Interest in phosphorylase (rational name: α-glucan orthophosphate glucosyl transferase) has not diminished. Madsen & Cori (116) compared the K_m of the phosphorylase reaction on glycogen with the binding constant obtained by studying the combination of phosphorylase and glycogen with the help of the ultracentrifugal separation method. The binding constant was about one order of magnitude larger than K_m. The authors tentatively explain this result through steric hindrance by the relatively large enzyme molecule in a static, as compared to a dynamic, situation.

As Krebs *et al.* (117) have shown, the conversion of phosphorylase-*b* to phosphorylase-*a* proceeds according to the equation

$$4\ \text{ATP} + 2(\text{phosphorylase-}b) \rightarrow \text{phosphorylase-}a + 4\ \text{ADP}.$$

Fischer *et al.* (118) suggest that the phosphate introduced during this reaction is linked to O-seryl residues. By trypsin treatment, the bound phosphate is released in a soluble form. The mechanism by which the phosphorylation of phosphorylase-*b* is connected to the subsequent dimerization is still unknown. However, no diester bonds are formed in the conversion reaction; there is also no evidence that the four phosphate groups incorporated into the molecule act directly in the dimerization process by chelation of a metal ion.

Lee (119) reports on the properties of purified potato phosphorylase. A preparation homogeneous in the ultracentrifuge has been obtained with a mol. wt. of 207,000; the total P content was 2 moles per mole protein. It could be shown that these 2 moles P correspond to 2 moles pyridoxalphosphate and that no other phosphate compound exists in the molecule. Thus, the potato phosphorylase differs from muscle phosphorylase-*a* by the absence of serine-bound phosphate and from phosphorylase-*b* by not being dependent on AMP for activity. In the presence of ATP and Mg^{++}, phosphorylase-*b* kinase does not phosphorylate potato phosphorylase. The enzyme

could be freed of pyridoxalphosphate at pH 4; because of the acid lability of the enzyme, reactivation by added pyridoxalphosphate was not complete.

Cellobiose phosphorylase (rational name: cellobiose orthophosphate glucosyl transferase) has been discovered in two further cellulytic microorganisms (120, 121).

Shukla & Prabhu (122) have studied the biosynthesis of sucrose in sugar cane. They report on the presence of a sucrose phosphorylase in this material and claim that "the equilibrium constant of the enzyme is 5.6 and differs markedly from those reported," namely, for the reaction catalyzed by sucrose phosphorylase from bacteria. It should be kept in mind, however, that the equilibrium constant is a property of a reaction rather than of an enzyme.

Adenosine nucleoside phosphorylase (rational name: adenosine nucleoside orthophosphate ribosyl transferase) from *E. coli* strain B has been studied and a specific sulfate inhibition of the catalyzed reaction has been found (123).

Glycosyl transferases that do not act on phosphorus-containing substrates.—The so-called D-enzyme, originally discovered by Peat, Whelan & Rees (124), catalyzes the reversible disproportionation of maltodextrins. For example, maltotriose, is acted on to give glucose and maltopentaose as first reaction products, whereas a whole series of maltodextrins in addition to glucose appears at equilibrium. Walker & Whelan (125) have now shown that, in the presence of a glucose-consuming system, e.g., hexokinase, D-enzyme brings about amylose formation. As the complete system is known to be present, e.g., in potatoes, the authors discuss the possibility that it may play a role in amylose synthesis in plants.

A rumen strain of *Streptococcus bovis* produces a dextransucrase, catalyzing the formation of an unbranched polysaccharide containing exclusively $\alpha(1\rightarrow6)$-linkages (126). The acceptor specificity is surprisingly wide; maltose, isomaltose, glucose, galactose, α-glucosides, lactose, and cellobiose stimulate the enzyme activity and serve as alternative glucosyl acceptors. The enzyme must be considered constitutive, the presence of sucrose in the medium not being required for its formation. Dextransucrase from *Leuconostoc mesenteroides* can use raffinose as an alternative acceptor (127).

D-Fructosyl transferase action, as catalyzed by levansucrase from *Aerobacter levanicum*, has been reviewed by Hestrin (128).

Acyl Transferases and Related Enzymes

A very useful, up-to-date monograph on CoA, its acyl derivatives, and their role in cell metabolism has appeared in German [Decker (129)].

Phosphotransacetylase (rational name: acetyl-CoA orthophosphate acetyl transferase), until now thought to be present in bacteria only, has been found in green gram bean seeds (*Phaseolus radiatus*) (196). A simple way of preparing the enzyme from this source is described; this may be of practical use, as the enzyme may serve as an analytical tool in determining the ac-

tivity of the citric acid cycle in biological material. Fluoroacetylphosphate has been shown to act as a donor in the reaction catalyzed by phosphotransacetylase (42).

Much work has been performed recently on enzymes which, although catalyzing a transfer of an acetyl group, are to be distinguished from the acetyl transferases in the proper sense of the word, because, in contrast to those enzymes, they combine the acetyl group with the acceptor molecule through its methyl group rather than through its carboxyl group. To distinguish this mode of action by name, Dixon & Webb (130), in their splendid treatise, call enzymes that combine the carboxyl group of the acetyl moiety with the acceptor transacetylase, whereas enzymes similar to the "condensing enzyme" should be termed transacetases. In the rational nomenclature, the corresponding terms are to be acetyltransferase and acetotransferase, respectively.

It now seems to be established that, in the synthesis of citrate by the citrate-condensing enzyme (rational name: acetyl-CoA oxaloacetate acetotransferase), the keto form of oxaloacetic acid reacts with the acetyl residue of acetyl-CoA [Englard (131); cf. also Bové *et al.* (132)].

Dixon & Kornberg (133) have developed a sensitive spectrophotometric method for measuring the activity of malic synthetase (rational name: acetyl-CoA glyoxalate acetotransferase), an enzyme of an action very analogous to the citrate-condensing enzyme. In addition to several other enzymes of the glyoxalate cycle, a malic synthetase has been found in enzyme preparations from peanut and sunflower cotyledons (134).

Rudney & Ferguson (135, 136) have summarized their work on a yeast enzyme that synthesizes β-hydroxy-β-methyl-glutaryl-CoA from acetyl-CoA and acetoacetyl-CoA. The authors call it the HMG-condensing enzyme; the rational name would be acetyl-CoA acetoacetyl-CoA acetotransferase. The equilibrium of the reaction catalyzed by the enzyme seems to be very much in favor of synthesis. The authors discuss the function of the enzyme in isoprenoid biosynthesis; Lynen *et al.* (137), who have confirmed most of the results of Rudney & Ferguson, suggest an additional role of the enzyme in the biosynthesis of acetoacetate via a so-called β-hydroxy-β-methylglutaryl-CoA cycle.

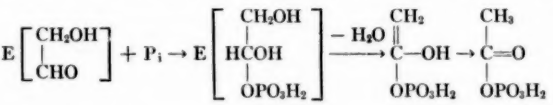
An enzyme transferring acyl groups, the action of which seems to be more complex, is responsible for the formation of δ-aminolevulinic acid in photosynthesizing bacteria. Succinyl-CoA condenses with a pyridoxalphosphate derivative of glycine to form δ-aminolevulinic acid and CO_2. A sixty- to eightyfold purification of the enzyme has been achieved, and its specificity, sensitivity against inhibitors, and kinetics have been studied [Kikuchi *et al.* (138, 139)].

Although the donor molecules do not contain preformed acetyl residues, the action of the so-called phosphoketolases must be considered an acetyl transfer. The action of the first-known enzyme of this group, the so-called

xylulose-5-phosphate phosphoketolase from *Lactobacillus plantarum*, can be represented by the equation (140)

D-xylulose-5-phosphate + P_i → D-glyceraldehyde-3-phosphate + acetyl phosphate.

A new enzyme of a similar action has been discovered by Schramm, Klybas & Racker (141) in *Acetobacter xylinum*. It cleaves fructose-6-phosphate phosphorolytically to acetyl phosphate and erythrose-4-phosphate; in addition, it also catalyzes the phosphorolytic breakdown of xylulose-5-phosphate in the same manner as the phosphoketolase from *Lactobacillus plantarum*. Ribulose-5-phosphate, sedoheptulose-7-phosphate, xylulose, fructose, hydroxypyruvate, and pyruvate are not attacked, however. As with the enzyme from *L. plantarum*, the phosphoketolase from *Acetobacter* also seems to require thiamine pyrophosphate and Mg^{++} for its action. When arsenate is substituted for phosphate, acetate and erythrose-4-phosphate are formed. The authors envisage the intermediate formation of an enzyme-glycolaldehyde complex which, under the influence of orthophosphate, reacts in the following manner:



Two further newly discovered enzymatic reactions show—at least formally—a certain analogy to the phosphoketolase reaction. In *E. coli* extracts, the synthesis of 2-keto-3-deoxy-D-*arabo*-7-phosphoheptonic acid from phosphoenolpyruvate and D-erythrose-4-phosphate proceeds according to the equation:

phosphoenolpyruvate + erythrose-4-phosphate + H_2O
→ 2-keto-3-deoxy-D-*arabo*-7-phosphoheptonic acid + P_i.

The enzyme responsible for this reaction has been found independently in two laboratories (142, 143); it seems to be absolutely specific for both substrates. The reaction is probably irreversible; in contrast to the phosphoketolase reactions, thiamine pyrophosphate is not required. Formally, the reaction equation suggests a transfer of the pyruvyl moiety from phosphoenolpyruvate to erythrose-4-phosphate; it is, however, the methylene group of phosphoenolpyruvate rather than the carboxyl group that combines with the acceptor molecule. In analogy with the similar situation with citrate-condensing enzyme, the name pyruvotransferase would be suggested for such an enzyme.

In extracts from *Pseudomonas aeruginosa*, Levin & Racker (144) have discovered a very similar reaction. 2-Keto-3-deoxy-8-phosphooctonic acid is formed from either ribose-5-phosphate or arabinose-5-phosphate and phosphoenolpyruvic acid, obviously by a pyruvotransferase reaction.

Although the group transferred by transketolase is glycolaldehyde rather than an acyl group, this enzyme is certainly closely related to those treated

in the last paragraphs. Transketolase requires thiamine pyrophosphate for its action, and Datta & Racker (145) now present evidence that, in the course of the action of the enzyme, an active glycolaldehyde-enzyme intermediate is formed, from which the ketol group is transferred to a suitable acceptor aldehyde. Thiamine pyrophosphate seems to be a part of the intermediate complex. On denaturation, the complex yields free glycolaldehyde.

Methyltransferases

An enzyme that catalyzes the transfer of methyl from S-adenosylmethionine to the ring-N of histamine has been found and partially purified by Brown *et al.* (146). The product of the enzyme action is 1-methyl-(β-aminoethyl)-imidazole (1-methylhistamine). The enzyme is not inactivated by versene; this demonstrates that it is different from the catechol O-methyl transferase [Axelrod & Tomchick (147)]. The histamine-methylating enzyme has been found in most tissues of all species studied so far and seems to play an important role in histamine metabolism.

In *E. coli* and various other microorganisms, an enzyme has been found that catalyzes the methyl transfer from S-adenosylmethionine to the 2-amino group of various aminopurines [Remy (148)].

Dimethylthetin homocysteine methyltransferase from rat liver has been purified 100-fold and the properties of the enzyme have been studied by Fromm & Nordlie (149). A survey of the distribution of the enzyme (150) revealed that it is present in appreciable amounts in the liver of all vertebrates tested; smaller amounts are found in kidney. The enzyme activity in green plants, moulds, yeast, and invertebrates is negligible. Various similarities in the occurrence, and also in the properties, of this enzyme and betaine homocysteine methyltransferase suggest a possible identity of these two enzymes.

Formyl-, Formimino-, and Hydroxymethyltransferases

Hartman & Buchanan (151) have studied the two steps by which formate is transferred from formyl derivatives of tetrahydrofolic acid (THFA) to precursors of inosinic acid in the course of the biosynthesis of this substance by purified enzyme preparations. In the absence of the so-called cyclohydrolase, an enzyme catalyzing the intraconversion of 10-formyl-THFA and 5,10-anhydroformyl-THFA, it was found that 5,10-anhydroformyl-THFA is the specific donor in the formylation of 2-amino-N-ribosylacetamide-5′-phosphate (glycinamide ribonucleotide), whereas the formyl residue for the formylation of 5-amino-1-ribosyl-4-imidazolecarboxamide-5′-phosphate derives from 10-formyl-THFA. The authors present a useful scheme illustrating the role of the various compounds in most known C_1-transfer reactions (see Figure 1).

The liver enzyme preparation of Miller & Waelsch (152) which carries out the deformimination of formimino-L-glutamate according to the equation

$$\text{formimino-L-glutamate} + \text{THFA} \rightarrow \text{glutamate} + \text{10-formyl-THFA} + NH_3$$

has been further studied by Tabor & Wyngarden (153). It was found that the overall reaction—a step in the degradation of histidine—represents the sum of three separate enzymatic reactions, the first of which is the actual transfer process:

formimino-L-glutamate + THFA → 5-formimino-THFA + L-glutamate.

The enzyme responsible for this reaction, the formimino-L-glutamate THFA formiminotransferase, has been purified 700-fold from hog liver. The steps following this reaction are: first, a deamination of 5-formimino-THFA to 5,10-anhydroformyl-THFA and ammonia, catalyzed by an enzyme called

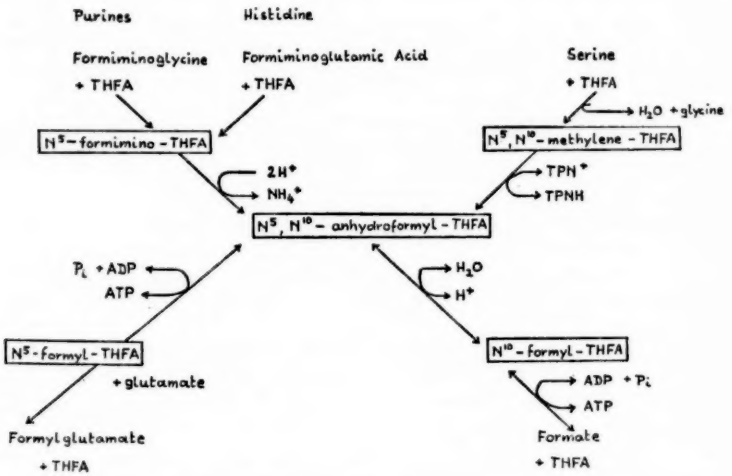


FIG. 1. The role of tetrahydrofolic acid derivatives in C_1-transfer reactions.

by the authors formimino-THFA cyclodeaminase; secondly, the hydrolysis of the anhydroformyl compound to 10-formyl-THFA, for which cyclohydrolase is responsible.

Blakley (154) has found that synthetic 5,10-anhydro-formyl-THFA differs from the product obtained through the enzymatic reaction of serine and THFA only by being a racemate. The same author studied the equilibrium of the reaction.

5,10-anhydroformyl-THFA + glycine ⇌ serine + THFA

spectrophotometrically and found it to lie to the left, the constant being about 0.3. Pyridoxalphosphate is firmly bound to the enzyme catalyzing this reaction; it may be assumed that, prior to their interconversion, serine and glycine react with this coenzyme to become "activated" for the enzyme ac-

tion. The same enzyme, which occurs in mammalian and avian liver as well as in ascites tumor, has been studied by Mell *et al.* (155) who also report that sarcosine can be oxidized in their enzyme preparations. It then serves as a hydroxymethyl donor according to the equation

N-hydroxymethyl glycine + THFA → 5,10-anhydroformyl-THFA + glycine.

Aminotransferases

Grein & Pfleiderer (156) have purified L-alanine α-ketoglutarate aminotransferase from pig heart. The purest preparation showed a turnover number of 32,000 per 100,000 gm. protein. The prosthetic group is tightly bound to the protein; the maximum pyridoxalphosphate content of the enzyme is 0.9 moles per 100,000 gm. of protein. The participation of —SH groups and of metal ions in the enzyme reaction is suggested.

L-Aspartate α-ketoglutarate aminotransferase has also been prepared from pig heart in a high state of purity (157). The enzyme contains 2 moles pyridoxalphosphate per mole (mol. wt. 110,000 ± 11,000). Jenkins & Sizer (158) also report kinetic and inhibitor studies with the purified enzyme. Kun *et al.* (159) have found an inhibition of aspartate α-ketoglutarate aminotransferase by α-keto-β-fluorosuccinic acid (fluorooxaloacetic acid). The inhibitor, which itself is very slowly aminated to fluoroaspartic acid, seems to compete with oxaloacetate for its site on the enzyme.

The role played by γ-aminobutyric acid in brain [cf. Elliott (160)] has been the reason for investigations of the metabolism of this compound. Baxter & Roberts (161) have devised a new method for the measurement of the activity of γ-aminobutyrate α-ketoglutarate aminotransferase based on the observation that α-amino acids chelate copper strongly, whereas the chelation with γ-amino acids is negligible. The γ-aminobutyrate α-ketoglutarate aminotransferase of brain requires pyridoxalphosphate for activity and is inhibited by sulfhydryl reagents. By histochemical methods, Salvador & Albers (162) have studied the localization of this enzyme in the nervous system of the monkey. The enzyme is largely confined to the gray matter of the central nervous system and, in general, exhibits progressively increasing activity in subcortical and lower structures. The occurrence of an enzyme with the same action in *Aspergillus fumigatus* had been reported some years ago (163); in addition, γ-aminobutyrate α-ketoglutarate aminotransferase activity has been found in *E. coli* (161) and in *Pseudomonas fluorescens.* Scott & Jakoby (164) have purified an enzyme preparation from the latter organism. Pyridoxalphosphate seems to be tightly bound to the enzyme protein; a kinetic study of the enzyme reaction is reported.

Nishizuka *et al.* (165) have partially purified an enzyme from *Pseudomonas* sp., that catalyzes a reversible transamination from β-alanine to pyruvate, resulting in the formation of stoichiometric quantities of malonic semialdehyde and α-alanine. In place of β-alanine, γ-aminobutyric acid can also act as amino donor.

An enzyme from *E. coli* catalyzes the amino transfer from N-succinyl-α-

amino-ϵ-ketopimelic acid to α-ketoglutarate with the formation of N-succinyl-L-diaminopimelic acid and glutamic acid, and it has been purified by Peterkofsky & Gilvarg (166). The reaction is reversible; the 150-fold purified enzyme is specific for glutamic acid as the amino donor and does not utilize DL-N-succinyl-*meso*-diaminopimelic acid as a substrate in the back reaction. The same activity has also been observed in various other bacteria.

Carbamyltransferases

The enzyme converting ornithine to citrulline from *Streptococcus lactis* has been purified by Ravel *et al.* (167). A previous investigation had shown that biotin deficiency in the organism is associated with the loss in ability to transfer the carbamyl moiety of carbamylphosphate to ornithine. Therefore, the biotin content of the enzyme fractions has been determined but biotin is not to be a component of the enzyme. The authors suggest that the vitamin exerts its action during the synthesis of the enzyme.

Sallach (168) has found an enzyme in normal liver and in Ehrlich ascites tumor, which catalyzes the carbamyl transfer from carbamylphosphate to hydroxyaspartic acid. The product of this reaction was identified as N-carbamyl-hydroxyaspartic acid. It may be, however, that this transcarbamylation is not caused by a new specific enzyme; the possibility that it is catalyzed by the already known carbamylphosphate aspartate carbamyltransferase cannot be excluded.

Sulfurtransferases

Westley & Green (169) report the preparation of a crystalline rhodanese (rational name: thiosulfate cyanide sulfurtransferase) from beef kidney. The crystalline beef kidney and beef liver enzymes are indistinguishable in specific activity, crystal form, and sedimentation rate. A similar study of the enzymatic properties of liver and kidney rhodanese of the same steer (170) seems to indicate the complete identity of the two enzymes.

Kun & Fanshier (171, 172) have continued their work on the enzyme catalyzing the transfer of sulfur from β-mercaptopyruvate to sulfite and also to cyanide. The enzyme was isolated and purified from liver. The catalytically active group that participates in the removal of sulfur from the substrate and in its transfer is believed to be a thiol-disulfide-copper system. The enzyme is found in all organs of the rat, the highest activity appearing in kidney and liver. Hylin & Wood (173, 174), studying the mechanism of this enzyme from rat liver, report that, in the absence of an adequate sulfur acceptor, a reactive form of sulfur is formed which decomposes at lower pH. The reactive sulfur has the properties of a non-diffusable polysulfide.

Coenzyme A-Transferases

A study of the mechanism of CoA transfer as catalyzed by acetoacetyl succinic thiophorase (rational name: acetoacetyl-CoA succinate CoA-trans-

ferase) has been made by Falcone & Boyer (175). The results obtained with ^{18}O-labeled succinate suggest an intermediate formation of mixed anhydrides in this reaction. In *Pseudomonas oxalicus*, Quayle & Keech (176) present evidence for an enzyme that catalyzes the reaction:

$$\text{acetyl-CoA} + \text{oxalate} \rightarrow \text{oxalyl-CoA} + \text{acetate}.$$

Sulfuryltransferases

While Roseman (177), was justified in writing a year ago that there had been no report of a sulfate transfer to known carbohydrate derivatives, several papers on such reactions have appeared during the past year. It seems to be established that adenosine-3′-phosphate-5′-phosphosulfate acts as the sulfate donor for the synthesis of sulfated mucopolysaccharides in mast cell tumors (178, 179), in rat liver (180, 181), and in the isthmus of hen oviduct (182). From the last material, an enzyme has been partially purified and its acceptor specificity has been investigated (183). It seems that only higher molecular polysaccharides are sulfurylated by the enzyme, whereas UDP-acetylgalactosamine cannot act as an acceptor.

Nose & Lipmann (184) present evidence for the existence of a whole group of "sulfokinases," i.e., sulfuryltransferases. It was shown that the sulfate transfer from adenosine-3′-phosphate-5′-phosphosulfate to phenols and steroids is attributable to different enzymes. But even the sulfurylation of the various steroids seems to be caused by more than one enzyme; the existence of two different enzymes, one specific for dehydroandrosterone and the other for estrone, could be established. The authors consider it probable that even more specific steroid sulfuryltransferases exist. Steroid sulfurylating enzymes are found in liver exclusively, whereas kidney and intestine contain only enzymes sulfurylating phenols.

Although tyrosine-O-sulfate is a normal excretory product, tyrosine is not sulfurylated by an enzyme system from liver. In this preparation, however, sulfate is transferred to tyrosine derivatives in which the carboxyl group is either absent or substituted, while the amino group is unsubstituted (185).

The formation of sulfamates is also catalyzed by an enzyme from liver, adenosine-3′-phosphate-5′-phosphosulfate acting as the sulfate donor (186). In the case of a liver enzyme from rat, but not from guinea pig, the sulfurylation of 2-naphthylamine to 2-naphthylsulfamate is strongly activated by steroids with an oxo group in 17-position. An intermediate formation of a steroid-17-enolsulfate is offered for an explanation of this effect.

Kaji & McElroy (187) have found that a cell-free extract from *Aspergillus sydowii* can catalyze the sulfurylation of choline. The enzyme responsible for this action is different from that sulfurylating nitrophenol in the same organism.

Miscellaneous

In cell-free preparations from rat brain, Brady *et al.* (188) have found an enzyme that catalyzes the formation of dihydrosphingosine from palmitic aldehyde and serine. CO_2 is split off during the reaction.

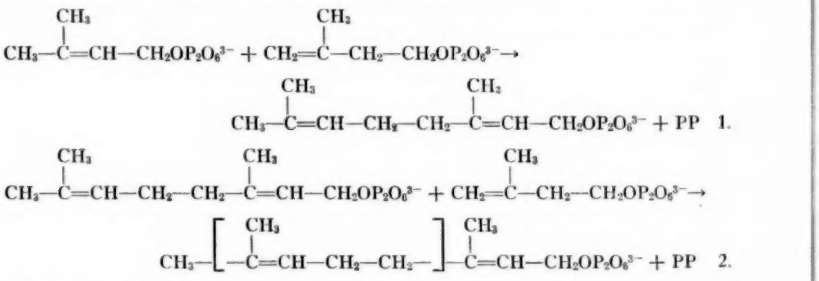
During this reaction, serine forms a complex with pyridoxal phosphate and Mn^{++} in which the methylene group at carbon atom 2 of serine is activated and then can participate in a carbon-carbon addition reaction. The process catalyzed by the enzyme can be represented by the equation

$$\text{palmitic aldehyde} + \text{serine} \xrightarrow{\text{pyridoxalphosphate, } Mn^{++}} \text{dihydrosphingosine} + CO_2.$$

Accordingly, the reaction can be considered an ethanolamine transfer.

Leder (189) has purified an enzyme from yeast which catalyzes the synthesis of thiamine monophosphate from 2-methyl-4-amino-5-hydroxymethylpyrimidyl pyrophosphate and 4-methyl-5-(β-hydroxyethyl)-thiazole phosphate. Pyrophosphate is split off during the reaction. Thus, the reaction is actually a transfer of the pyrimidyl moiety to the thiazole phosphate residue. A similar mechanism has also been postulated by Camiener & Brown (43).

During the transformation of mevalonic acid to squalene by soluble enzyme systems from yeast, the active isoprene unit seems to be 3-isopentenylpyrophosphate [Lynen *et al.* (190, 191)], which is in an enzyme-catalyzed equilibrium with its isomer, γ,γ-dimethylallylpyrophosphate [Agranoff *et al.* (192)]. Evidence has been presented for the existence of an enzymatic mechanism which catalyzes the condensation of 3-isopentenylpyrophosphate and γ,γ-dimethylallylpyrophosphate to give geranylpyrophosphate and pyrophosphate. In a second step—probably catalyzed by the same isopentenyl transferase—farnesylpyrophosphate is formed from isopentenylpyrophosphate and geranylpyrophosphate.



The existence of the same mechanism has also been demonstrated in rat liver (191).

It seems probable that the isopentenyl transfer mechanism plays a major role in the formation of all isoprenoid natural compounds, isopentenyl-

pyrophosphate being the long-sought common building stone postulated by Ruzicka (192a).

Results from several laboratories have for some time suggested a function of biotin in certain carboxylation processes, although no information on the mode of action of biotin in these reactions has been available. Lynen and coworkers (193) now present clear-cut evidence on the coenzyme function of biotin. When studying the carboxylation of β-methylcrotonyl-CoA to β-methylglutaconyl-CoA with an enzyme preparation of a *Mycobacterium* sp. grown on a medium containing isovalerianic acid as the only carbon source, they found that the enzyme carrying out the reversible reaction

$$\text{ATP} + \text{CO}_2 + \beta\text{-methylcrotonyl-CoA} \rightleftharpoons \beta\text{-methylglutaconyl-CoA} + \text{ADP} + \text{P}_i$$

contains biotin as a prosthetic group. By virtue of tracer experiments, the authors postulate the following reaction sequence:

$$\text{ATP} + \text{biotin-enzyme} \overset{Mg^{++}}{\rightleftharpoons} \text{ADP} \sim \text{biotin-enzyme} + \text{P}_i \qquad 1.$$

$$\text{ADP} \sim \text{biotin-enzyme} + \text{CO}_2 \overset{Mg^{++}}{\rightleftharpoons} \text{CO}_2 \sim \text{biotin-enzyme} + \text{ADP} \qquad 2.$$

$$\text{CO}_2 \sim \text{biotin-enzyme} + \beta\text{-methylcrotonyl-CoA} \rightleftharpoons \beta\text{-methylglutaconyl-CoA} + \text{biotin-enzyme.} \qquad 3.$$

In the Reaction 3, β-methylcrotonyl-CoA can be replaced by free biotin; the authors succeeded in preparing free CO_2~biotin in this way. The nature of the bonding of carbon dioxide to biotin is not yet clear. Probably one of the two ureido nitrogen atoms of biotin is the point of attachment. The authors discuss the possibility that various other carboxylation processes, e.g., the carboxylation of propionyl-CoA to methylmalonyl-CoA or the carboxylation of acetyl-CoA to malonyl-CoA, are mediated by similar biotin-dependent mechanisms. Results obtained by Wakil (194, 195) seem to support this hypothesis, whereas Tietz & Ochoa (49) state definitely that their enzyme from pig heart, which catalyzes the carboxylation of propionyl-CoA, does not contain biotin.

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CHEMISTRY OF THE CARBOHYDRATES[1,2]

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This review continues the practice of discussing selected topics rather than attempting to cover in less detail the whole of the vast literature on carbohydrates. Simply to print the titles and references of papers on carbohydrate chemistry listed in *Current Chemical Papers* during 1958 would occupy more than twice the space allotted to this article.

Review articles published recently have dealt with the following topics: rare sugars [Bayne (1)], new sugars [Overend (2)], sugar epoxides [Newth (3)], sugars of *Actinomycetes* antibiotics [Van Tamelen (4)], seaweed polysaccharides [Hirst (5)], and the structural analysis of polysaccharides [Manners (6)]. In addition to the topics already listed (7), *Symposium I* of the *Proceedings of the Fourth International Congress of Biochemistry*, contains reviews on amino sugars [Kuhn (8)] and the enzymology of levans [Hestrin (9)]. The *Encyclopedia of Plant Physiology*, Volume 6, (10) contains articles by 36 contributors and deals fully with the structures, properties, and enzymology of plant and fungal carbohydrates. The chemistry, physical chemistry, and enzymology of cellulose and starch are covered in 12 review articles in *Recent Advances in the Chemistry of Cellulose and Starch* (11).

Nomenclature of Oligosaccharides

Reports of new oligosaccharides produced by chemical or enzymatic synthesis or by degradation of polysaccharides continue unabated, and the problem of applying convenient names to these compounds becomes increasingly urgent. Any oligosaccharide can be named in terms of the recommended nomenclature (12), but these names do not lend themselves to repetition. In consequence, the practice has been to apply a trivial name which is usually indicative of its native origin but gives no clue to its structure. The number of oligosaccharides is now far too big for one to maintain a mental link between the trivial name and the structure. To meet this situation, the reviewer devised a system of nomenclature of oligosaccharides to provide names indicative of structure, yet having some of the conciseness of a trivial name. It was begun in *The Biochemical Journal*, and it will be used here. The system accepts the use of trivial names for disaccharides. Its conventions can be explained in terms of examples, except that aldoses are assumed to be

[1] The survey of the literature pertaining to this review was concluded in September, 1959.

[2] The following abbreviations are used: tosyl for *p*-toluenesulphonyl; trityl for triphenylmethyl; UDP for uridine diphosphate; UDPG for uridine diphosphate glucose.

in the D series and to have pyranose rings and ketoses to be in the D series and to have furanose rings, unless otherwise stated. The use of -O- to indicate a glycosidic linkage is omitted. Thus, panose [O-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose] is 4-α-isomaltosylglucose or 6^2-α-glucosylmaltose; the superscript numeral indicates the serial number of the glucose unit of the maltose involved in substitution, the reducing unit always being no. 1. 1-Kestose is 1^F-β-fructosylsucrose; raffinose, stachyose, and verbascose are, respectively, 6^G-α-galactosyl-, 6^G-α-galactobiosyl-, and 6^G-α-galactotriosyl-sucrose.[3] (Galactobiose is currently used for 6-α-galactosyl-galactose, although the use of a generic name for a specific sugar is unsatisfactory.) A pentasaccharide obtained from an α-amylolysate of amylopectin (13) is 6^3-α-maltosylmaltotriose. The fully systematic name is O-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-D-glucose. Kuhn's fucosidolactose (8) is 2^{Gal}-α-L-fucosyl-lactose.

Oxidations with Periodate

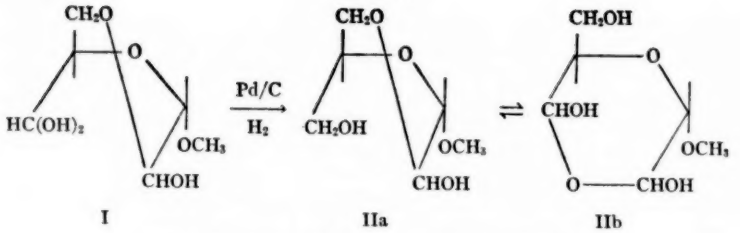
The oxidation of α-glycol groups with periodate or, less frequently, lead tetraacetate is nowadays the most widely used means of structural determination of carbohydrates. In most cases, the concern is to measure the amount and rate of formation of the simple end products of the oxidation—formic acid, formaldehyde, carbon dioxide, and ammonia—and the consumption of oxidant. Alternatively, the carbohydrate skeleton remaining after these products have been split off can be examined. Much work is being done, and still remains to be done, to clarify the mechanism of periodate oxidation and to improve the conditions of its use. The most important factor bearing on the course of oxidation is the pH of the medium. In unbuffered periodate, aldoses are oxidized in their ring forms, and attack is directed at the hemiacetal α-glycol group (14 to 17). The resulting formyl esters are extremely stable at this pH, and complete oxidation must wait for their hydrolysis. Fructose has two α-glycol groups involving the C-2-hemiacetal hydroxyl and, in periodic acid, both sites, at C-1—C-2 and C-2—C-3, are attacked (18). Polyols in periodic acid are attacked preferentially at *trans*-α-glycol groups, but the freed aldoses are attacked more rapidly than the parent polyol (15).

Barker & Shaw (19) noted a rapid uptake of one molecule of periodate at pH 7 by sugars containing a *cis-cis*-1,2,3-triol grouping and proposed the formation of a tridentate complex, a view challenged by Nevell (20). This uptake of oxidant is observed if periodate is measured by the arsenite-bicarbonate method of Fleury & Lange (21), but not if acid-iodide is used. Periodate can, therefore, become non-oxidative towards arsenite without having oxidized the sugar, and lack of awareness of this could lead to errone-

[3] The use of names such as maltosylsucrose and cellobiosylsucrose to describe certain analogues of sucrose (9) is misleading, since the names convey the impression of tetrasaccharides, whereas the sugars in question are trisaccharides, 4^G-α-glucosyl-sucrose and 4^G-β-glucosylsucrose, respectively.

ous estimates of oxidant consumption. Since arsenite is still commonly used for estimating periodate, attention is drawn to two further defects: (*a*) If the oxidation medium is more acid than pH 7, the change in pH occasioned by the addition of bicarbonate, which precedes that of arsenite, may initiate oxidation. This was noted in 1941 by Van Slyke *et al.* (22) and accounts for some anomalies in glucose oxidation noted in 1948 by Hughes & Nevell (23). (*b*) The arsenite is titrated with iodine; if the carbohydrate oxidation products contain activated hydrogen atoms, iodine is taken up, again erroneously indicating too high a consumption of periodate. This was noted by Schwarz & MacDougall (24, 25) with benzyloxymalondialdehyde formed in the oxidation of 2-O-benzyl-D-arabinose. Cleaver *et al.* (26) have recently noted a similar reaction between iodine in bicarbonate and periodate-oxidized 2-deoxy sugar derivatives. The hydrogen atoms at the deoxy center are activated by the adjacent aldehyde group, and an iodinated compound was isolated when periodate-oxidized 1-(2-deoxy-D-galactosyl)benzimidazole was treated with iodine-bicarbonate. Anomalous uptakes of periodate were noted recently in the oxidation of glucose-6-phosphate (27) and 3-aminopentofuranosyl derivatives (28). The arsenite method was used in both cases and they might profitably be re-examined by the acid-iodide method, to which these objections do not apply.

Structural examination of the dialdehyde products of oxidation of simple glycosides helps in the understanding of the behaviour of oxidized polysaccharides. Smith and co-workers have made extensive studies of these compounds. They conclude that the aldehydic groups show a strong tendency to hydrate or to undergo cyclization. Both effects occur with oxidized methyl α- and β-glucopyranoside and are detected by the behaviour of the dialdehydes on reduction (29). The oxidized methyl α-glucoside I is hydrogenated with a palladium-charcoal catalyst only at its hydrated aldehydic group, forming the tautomeric structures IIa and IIb (see Figure 1). A Raney nickel catalyst causes reduction of both aldehydic groups. Structures I, IIa, and IIb, derivatives of 1,4-dioxane, are quite stable to acid but, when fully reduced, become extremely acid-labile. The dialdehydes from the methyl α-glycosides of L-rhamnose, L-arabinose, and 6-O-methyl-D-galactose do not have primary hydroxyl groups and cyclize as in formula III (oxidized α-L-rhamnoside) to compounds inert to reduction with palladium-charcoal (29, 30). All form cyclic hemiacetals with methanol or methanol-hydrogen chloride as do polysaccharide polyaldehydes, which also undergo cross-linking between chains (31, 32). Hydrated glycogen polyaldehyde is probably cross-linked (33). Goldstein, Lewis & Smith (32) and Guthrie & Honeyman (34) have studied the dialdehyde from methyl 4,6-O-benzylidene-α-D-glucoside. This crystallizes as the dihydrate IV and loses both molecules of water on sublimation. Both hydrated and anhydrous forms are thought to exist in equilibrium in water (34). The ease of oxidation of the 4,6-O-benzylidene derivatives of the methyl aldohexopyranosides has been correlated with ring conformation and the relative disposition of the hydroxyls at C-2 and C-3



I IIa IIb

FIG. 1. Reduction of the hydrated aldehyde (I) and tautomeric cyclic reduction products (II, a, b).

[Honeyman & Shaw (35)]. The methyl α-D-altroside derivative has axial hydroxyls at these positions and is not oxidized, since a planar cyclic periodate ester cannot be formed.

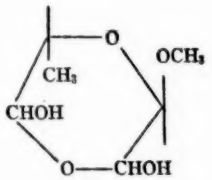
Garner *et al.* (36) found that the aglycone group of phenyl D-glucopyranoside inhibits oxidation at C-2—C-3 in the β-anomer but not in the α-anomer, and the trityl group in methyl 6-O-trityl-α-D-glucoside hinders C-3—C-4 oxidation.

Smith and co-workers have described three methods whereby polysaccharide polyaldehydes can yield structural information. Each method involves reduction of the aldehyde to the polyalcohol; sodium borohydride, like Raney nickel, reduces every aldehydic group. The three methods are as follows:

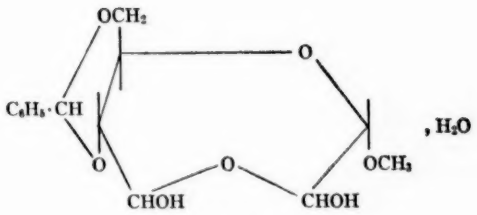
(*a*) The polyalcohol is completely hydrolysed with acid. Taking starch and glycogen as examples (37, 38), terminal non-reducing end groups yield glycerol from C-4—C-6 of the original glucose unit, while erythritol is formed from internal 1,4- or 1,4,6-substituted glucose units (C-3—C-6), and glycolic aldehyde (C-1—C-2) from all these units. The glycerol:erythritol ratio therefore measures the non-reducing end group content. Glucose has also been found in the hydrolysates, which may possibly be indicative of 1→3-links. This also occurs with cellulose (39).

(*b*) The polyalcohol is methylated, hydrolysed, and the products identified. This gives more information than method (*a*), since positions of substitution in the original polysaccharide are indicated by the location of the free hydroxyl groups of the fragments. Cellulose, for example, gives 1,4-di-O-methyl erythritol and methoxyacetaldehyde (40); amylopectin gives D-(1-O-methylerythritol) from 1,4,6-substituted glucose units (41). The polyaldehydes from the methyl glycosides of D-xylose, D-glucose (42), cellobiose, and lactose (43), have been studied as model substances for later work on polysaccharides.

(*c*) The polyalcohols are extremely labile to acid and can be split without rupturing the glycosidic bonds between unoxidized parts of the molecules. Figure 2 shows the decomposition on oxidation of a β-D-glucan linked alternately 1→4 and 1→3. The products are glycolic aldehyde and D-(2-O-β-D-



III



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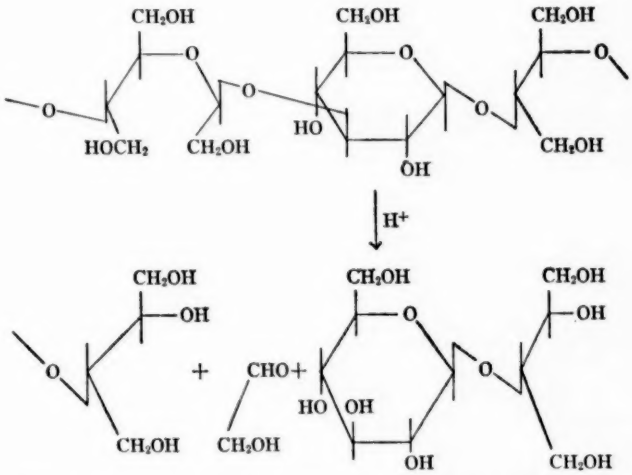


FIG. 2. Decomposition by cold dilute acid of periodate-oxidized glucan.

glucosyl erythritol). Goldstein *et al.* (44) have applied the method to oat β-glucan, from which erythrityl β-D-glucopyranoside, β-laminaribioside, and β-laminaritrioside were obtained. This confirms the report by Peat, Whelan & Roberts (45) of β-1→4- and β-1→3-links in this polysaccharide but, contrary to this report, proves that 1→3-links occur in sequence.

Moyer & Isbell (46) have described an alternative to method (*a*) to improve on the low recovery (65 per cent) of the products. The polyaldehyde was hydrolysed with sulphurous acid. Two of the products from oxidized dextran, glucose, and erythrose, were estimated by reaction with $Na^{14}CN$. It is stated, however, that the procedure is lengthy and requires specialized techniques and equipment.

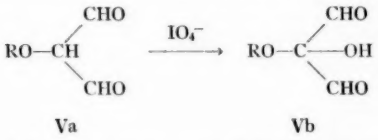
Sulphurous acid, under pressure at 90–115°, has also been used by Van Cleve & Mehltretter (47) to hydrolyse starch polyaldehyde. D-Erythrose and glyoxal were obtained in 80 and 90 per cent yields, respectively. Direct acid hydrolysis has long been known to give poor yields of these products. The success now achieved is attributed to the combination of sulphurous acid with the aldehyde groups, thus preventing them from forming 1,4-dioxane structures (see p. 107).

A quantitative oxidation of starch polyaldehyde is obtained with chlorous acid (48). Whistler, Chang & Richards and O'Meara & Richards showed that the aldehyde is degraded by lime water or sodium hydroxide to DL-2,4-dihydroxybutyric, glycolic, and formic acids, and these are also formed from cellulose polyaldehyde (49, 50). From dextran and corncob xylan polyaldehydes, the products are predominately glycolic and lactic acids (51).

Unrau & Smith (52) noted that periodate will liberate formaldehyde from the reducing-end group (reduced by borohydride) of a polysaccharide without forming the aldehyde from the untreated polysaccharide. The proportion of reducing ends is thereby estimated. Anderson *et al.* (53) have used a similar method to measure the degree of polymerization of laminarin. Parrish & Whelan (54) noted that, at pH 2.0, the rate of periodate over-oxidation of maltodextrins is directly proportional to their content of reducing-end groups. Comparison of the rates of over-oxidation of synthetic amyloses with that of a pure maltodextrin enabled the degree of polymerization of each amylose to be calculated. The results were in good agreement with the known values of the degree of polymerization of the amyloses.

The over-oxidation reaction referred to is the oxidation of the malondialdehyde structure Va (see Figure 3) formed after the normal α-glycol oxidation of the reducing-end groups of 1→2-, 1→3-, and 1→4-linked hexose (and other) oligo- and poly-saccharides. Over-oxidation results in a progressive erosion and final destruction of the whole molecule. Cantley, Hough & Pittet (55) have studied the details of the reaction and find that it divides after the formation of the hemi-acetal Vb, some of which is hydrolysed before being further oxidized [see also Head (56)].

Using very dilute periodate (0.4 m*M*), Clancy & Whelan (57) found that the reducing groups of glucose disaccharides underwent preferential oxida-



Va Vb

FIG. 3. Oxidation of the malondialdehyde structure.

tion, the preference persisting when the reducing group was converted into sorbitol or gluconic acid. The rate of oxidation was characteristic of the glucosidic linkage.

ACID REVERSION

Acids catalyse the dehydration of monosaccharides in three ways: (*a*) intermolecular condensation to form oligo- and poly-saccharides, (*b*) intramolecular elimination of water to form anhydrides, and (*c*) formation of unsaturated furfural derivatives by more extensive loss of water. Reaction (*c*) is irreversible and occurs most readily with ketoses (58). This discussion is confined to reactions of aldoses. Reactions (*a*) and (*b*) are termed acid-reversion reactions and are of interest for their preparative potentialities and as the cause of formation of artefacts during linkage analysis of polysaccharides.

Formation of polysaccharides.—In order to cause the greatest degree of polymerization of monosaccharides, it is necessary to exclude water. In dimethyl sulphoxide with dry hydrogen chloride at room temperature for five days, glucose gives 4 per cent of products which are non-diffusible through cellophane (59). Much greater yields of such material (about 60 per cent, 7000 to 30,000 M) are obtained by heating glucose at 140–170° in a vacuum with 0.164 per cent phosphorous acid (60, 61). The synthetic polymers are highly branched and have been sulphated for use as a blood anticoagulant (62). Similar substances are formed by a boric acid catalyst (63), and 1→6- and 1→4-links, mainly of α-configuration, predominate. The reducing-end groups are perhaps masked by anhydride formation.

Preparation of oligosaccharides and anhydrides.—When aqueous solutions of aldose monosaccharides are treated with acid, the polymerization is limited to the formation of oligosaccharides and anhydrides. For preparative purposes, the sugar concentration should be high and the treatment with hot dilute, or cold concentrated acid. Linkage types found in synthetic disaccharides isolated in recent studies are: from D-xylose, α-1→2, α-1→3, α-1→4, β-1→4, and ββ (trehalose type) (64); from D-arabinose, ββ (65); from L-arabinose, β-1→3, β-1→4, and ββ (66); from D-mannose, α-1→6, β-1→3, β-1→4, and β-1→6 (66); from D-glucose, α-1→3, α-1→4, α-1→6, β-1→2, β-1→3, β-1→4, and β-1→6 (67). Where the ring systems have been identified, they are pyranose. In products from aldohexoses, the 1→6-link predominates, and the reaction with glucose can be made to give good yields of isomaltose

(α-1→6) and gentiobiose (β-1→6) (68). Another practical route to the latter sugar is by hydrogenation of amygdalin (69). Hydrol, the syrupy residue remaining when glucose is crystallized from acid hydrolysates of starch, is a convenient source of reversion products, and at least 10 of the glucose disaccharides have been obtained from it (70 to 72). N-Acetyl-D-glucosamine exposed to moist hydrogen chloride forms the α- and β-1→6-disaccharides (73).

Accompanying the disaccharides are the sugar anhydrides, formed by intramolecular elimination of water between C-1 and C-6. These can be in the pyranose or furanose modifications (74), and their formation has been extensively studied by Richtmyer and co-workers (75). The percentage yields of such compounds formed by heating dilute solutions of D-aldohexoses in acid are: idose (75), altrose (57), gulose (43), allose (14), talose (12), galactose (1.7), mannose (0.6), and glucose (0.2 to 0.3). The absence of a hydroxyl group at C-3 helps anhydride formation; 3-deoxy-D-mannose and -D-glucose form 29 and 10 per cent, respectively, of anhydride (76). 1,6-Anhydro-D-glucopyranose (levoglucosan) polymerizes readily at 120° in presence of monochloroacetic acid (77). Polymers larger than 300,000 M are formed. A polymer is formed simply by heating the levoglucosan at 235–240° for 15 min. and contains α-1→6-, β-1→6-, α-1→4-, β-1→4-, and β-1→2-bonds (78).

Another reaction not yet considered is epimerization by acid. Hough & Pridham (79) found that 0.1m*N*-acid converts L-arabinose into ribose and erythropentulose; oligosaccharides were formed only at higher concentrations of acid.

A most interesting biological effect of an acid reversion product was noted by Slaminski *et al.* (80). Glucose or starch heated in acid gave mixtures of oligosaccharides that stimulated the adaption of bakers' yeast to respiration. Acid hydrolysis of this fraction gave 2,5-anhydro-L-idose, which was also active towards yeast. Dekker & Hashizume (81) recently prepared this sugar by mild acid hydrolysis of 5,6-anhydro-1,2-O-isopropylidene-D-glucofuranose, contradicting an earlier claim that glucose was the only product. The anhydro-idose is itself acid-labile, forming 5-hydroxymethylfurfural 200 to 300 times faster than does glucose.

Reversion during linkage analysis.—Oligosaccharides, particularly disaccharides, obtained in low yield during the linkage analysis of polysaccharides by partial hydrolysis with acid may be acid-reversion products rather than structural fragments. Control experiments, usually performed by treating the appropriate monosaccharide in the same way, are, therefore, necessary. Peat *et al.* (82) quantitatively analyzed the products from glucose heated in 0.33 *N*-sulphuric acid. The major products were the sugar anhydrides (about 70 per cent of the total) and the 1→6-linked disaccharides (15 to 20 per cent). The percentage of glucose undergoing reversion was small (0.54 per cent after 2 hr. at 100°), but correspondingly small quantities of oligosaccharides could be isolated from polysaccharide hydrolysates. The amount of isomalt-

ose isolated from a laminarin hydrolysate (83) was exactly equal in yield (0.06 per cent) to that formed by acid reversion, and the sugar was judged to be an artefact. This type of control experiment is unsatisfactory since polysaccharide hydrolysis involves bond splitting and the energy liberated may assist a recombination reaction. Peat *et al.* (82) therefore suggest that the control be made with the disaccharide appropriate to the polysaccharide, e.g., laminaribiose for laminarin. Jones & Nicholson (66) make the ingenious suggestion that the appropriate radioactive monosaccharide be added before hydrolysing the polysaccharide. All oligosaccharides from the polymer will then be non-radioactive. Reversion is minimized by keeping the concentrations of polysaccharide and acid and the temperature of reaction as low as is practical.

Monosaccharides

3,6-Dideoxy aldohexoses.—Over the past few years, Westphal, Staub, and their colleagues have discovered four 3,6-dideoxy aldohexoses in the highly branched endotoxin lipopolysaccharides of *Salmonella* species and *Escherichia coli*, where they occur as acid-labile end groups, thereby determining the immunological specificity. A fifth sugar, ascarylose, was found by Lederer *et al.* in ascarylic alcohol, a liposoluble unsaponifiable component of the egg membrane of *Parascaris equorum*. The alcohol is a mixture of three crystalline ascarosides, containing one or two units of ascarylose (84, 85). The clue to the structures of these sugars was their characteristic behaviour on periodate oxidation (86). The ease with which 2,5-dideoxypentoses are formed in this reaction is a basis for their estimation (87). Three of the sugars have been synthesized by C-6-deoxygenation of the corresponding 3-deoxyhexoses. Abequose from *Salmonella abortus* is 3,6-dideoxy-D-galactose (88, 89), tyvelose from *S. typhi* is 3,6-dideoxy-D-mannose (88, 90), and paratose from *S. paratyphi* A is 3,6-dideoxy-D-glucose (90, 91). Colitose from *E. coli* O 111 is 3,6-dideoxy-L-galactose (92), and ascarylose is 3,6-dideoxy-L-mannose (88, 91). Five of the possible eight 3,6-dideoxy aldohexoses have, therefore, been found in nature, and a sixth, 3,6-dideoxy-L-talose, has been synthesized from L-rhamnose (91, 93).

Esters.—6-O-Acetyl-D-glucose is a major metabolite of *Bacillus megatherium*, which contains a deacetylase specific for this sugar (94, 95). The acetate was synthesized simply by heating glucose in 50 per cent acetic acid [see also (97)], a process which, when applied to polysaccharides, causes random acetylation of primary and secondary hydroxyls (96), as does formylation of amylose with 90 per cent formic acid (98). Periplanetin, from the solid excretion on the necks of *Blattidae*, is 1-O-β-benzoyl-D-glucose (99).

Mung bean (*Phaseolus aureus*) seedlings contain a wide range of sugar nucleotides and enzymes which act on them. Hassid and co-workers have reviewed work in this field (100) and have recently described the synthesis by mung bean enzymes of the 1-phosphate esters of α-D-galactose, α-D-galacturonic acid, D-glucuronic acid, and L-arabinose (101, 102). 1,5-An-

hydro-D-glucitol is phosphorylated at C-6 by yeast hexokinase, and the ester inhibits rabbit muscle phosphoglucomutase and phosphoglucose isomerase but not yeast glucose-6-phosphate dehydrogenase (103).

Isotopically labelled sugars.—Isbell *et al.* have made available ^{3}H-labelled aldonic acids, glycitols, and sugars (104) and have synthesized D-[3-^{14}C]glucose by ascent of the sugar series from D-glyceraldehyde, using the cyanohydrin reaction (105). D-[^{14}C]Mannitol is prepared in high yield by exposing fronds of *Fucus vesiculosus* to $^{14}CO_2$ (106).

Miscellaneous.—By chemical synthesis, D-apiose proved to be the naturally occurring open-chain isomer (107). The L-isomer has also been synthesized (108, 109). Davies (110) has recorded the paper-chromatographic mobilities of 12 of the 16 D-aldoheptose sugars. A diethylidene octose (111) and a 4-C-formyl-nonose (112) have been synthesized.

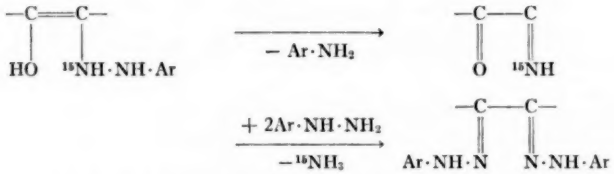


FIG. 4. Pathway for osazone formation. Ar = *p*-nitrophenyl.

D-Talose is the reducing-sugar component of hygromycin B (113). Noviose is 4-O-methyl-5,5-dideoxy-dimethyl-L-lyxose and occurs in novobiocin as the 3-O-carbamyl ester (114). The biosynthetic pathway of L-rhamnose from the rhamnolipid of *Pseudomonas aeruginosa* has been studied with ^{14}C-labelled substrates; D-[6-^{14}C]glucose gives rise exclusively to L-[6-^{14}C]rhamnose (115).

From three possible pathways of osazone formation, Bamdas *et al.* (116) chose that in Figure 4. ^{15}N-labelled *p*-nitrophenylhydrazones were treated with unlabelled *p*-nitrophenylhydrazine, and the fate of the ^{15}N was determined.

Alduronic acids epimerize with ease at pH 7 and 100° [Fischer & Schmidt (117)]. Epimerization, like that of aldonic acids, occurs next to the carboxyl group. After a few hours, equilibrium mixtures containing large amounts of the products are formed. The following conversions have been demonstrated: D-glucuronic acid and L-iduronic acid; D-mannuronic acid and L-guluronic acid; D-galacturonic acid and L-altruronic acid. The need for careful handling of uronic acids is evident, although Fischer & Schmidt think it unlikely that epimerization occurred during the isolation of L-iduronic acid from β-heparin (118) or of L-guluronic acid from alginic acid (119).

AMINO SUGARS

The widespread occurrence in nature of many amino sugars warrants their separation from other monosaccharides for a fuller review.

Sialic acids.—The enzymatic cleavage of N-acetylneuraminic acid to N-acetyl-D-mannosamine and pyruvic acid, by which means Comb & Roseman (120) first demonstrated the true nature of the component amino sugar, has been repeated with a *Vibrio cholerae* extract (121). Its receptor-destroying enzyme, which releases bound N-acetylneuraminic acid and other sialic acids, has been crystallized (122).

The structure of N-acetylneuraminic acid at C-1 to C-4 was not clearly defined in earlier work, but Kuhn & Brossmer (123) have made a chemical conversion of this part of the molecule into succinic acid and confirm that the conjectures of the structure were correct. A new method of estimating N-acetylneuraminic acid also depends on the release of C-1—C-4 as β-formylpyruvic acid by periodic acid; greater specificity is claimed (124).

Berggård & Odin (125) have studied the extreme acid and alkali lability of N-acetylneuraminic acid and other sialic acids. Maximum stability is at pH 4 to 5. Pyrrole 2-carboxylic acid was rigorously identified as an alkaline degradation product, but estimates of its amount simply by ultraviolet absorption (126) are incorrect.

Kuhn's original lactaminic acid-lactose from milk is 3^{Gal}-α-N-acetylneuraminosyl-lactose (127). The 6^{Gal}-isomer has now been obtained from milk (8), and the two are distinguished in that the receptor-destroying enzyme of *V. cholerae* splits the 3- but not the 6-isomer. In contrast, Gottschalk & Graham (128) find that mild alkali splits bovine salivary mucoprotein to 6-α-N-acetyl-neuraminosyl-N-acetyl-D-glucosamine, which is attacked by the receptor-destroying enzyme. This disaccharide is perhaps the sole prosthetic group of the mucoprotein. In orosomucoid, N-acetylneuraminic acid is an end group linked to C-3 of galactose (129).

Naturally occurring amino sugars.—Recently discovered amino sugars in antibiotics may be briefly described. Paromomycin consists of one molecule each of D-glucosamine, all-*trans*-2-deoxystreptamine, D-ribose, and a 2,6-diaminohexase; all linkage positions are known (130). Pseudoneamine is D-glucosamine linked to the same deoxystreptamine (131). This occurs again in kanamycin, where it is linked at C-4 and C-6, respectively, with 3-amino-3-deoxy- and 6-amino-6-deoxy-D-glucose (132). Kanamycin B differs in that it lacks the 6-amino sugar (133). Puromycin contains 3-amino-3-deoxy-D-ribose (134). Neomycin C contains 2,6-diamino-dideoxy-D-glucose in α-linkage with C-2 of D-ribose (135). Neomycin B is identical with C except that it contains a different diaminohexose of unknown structure (neosamine B).

Trehalosamine (α-glucosaminyl α-glucosaminide) occurs in a streptomycete (136) and D-fucosamine in the specific polysaccharide of *Chromobacterium violaceum* (NCTC 7917), along with glucosamine, glucose, galactose, and D-*glycero*-D-*manno*heptose (137). A polysaccharide from *Bacillus subtilis* contains a diamino-trideoxy hexose, characterized as the crystalline 4-acetamido-2-amino-2,4,6-trideoxy compound (138).

Just as Comb & Roseman had shown that mannosamine from N-acetylneuraminic acid had been mistaken for glucosamine (see above), so they

found it to be mistaken for galactosamine (139). Cardini & Leloir (140) had reported the formation of N-acetyl galactosamine (via the UDP-derivative) from UDP-N-acetyl glucosamine by a rat liver enzyme, but the product is now claimed to be N-acetyl mannosamine. Galactosamine is, however, formed by the same rat liver enzyme that interconverts UDP-galactose and UDP-glucose (141). This enzyme interconverts both UDP-glucosamine and UDP-N-acetyl glucosamine (142). A mixture of the latter with UDP-N-acetyl galactosamine, as obtained from hen oviduct, is a practical source of the galactosamine compound (143).

Chemical syntheses.—Amino sugar syntheses by the Amadori rearrangement have been reviewed by Pigman *et al.* (7). Kuhn, Bister & Fischer (144) have summarized the syntheses of all eight 2-amino-2-deoxy-D-hexoses by partial catalytic hydrogenation of amino acid nitriles. For example, D-lyxose is converted into N-phenyl-lyxosamine, which, with hydrogen cyanide, yields a mixture, separable by crystallization, of the 2-deoxy-2-phenylamino acid nitriles of D-galactosamine and D-talosamine. Hydrogenation gives the amino sugar hydrochloride and cyclohexanone (from the phenyl group). D-Lyxose can be conveniently prepared from D-galactose (145). The yields of amino sugars are generally good, but N-acetyl-D-mannosamine is an exception (1 per cent), and a 14 per cent yield is obtained on epimerization of N-acetyl-D-glucosamine (146). 2-Amino-2,6-dideoxy-L-glucose (L-quinovosamine) was also obtained by the nitrile method (147) and the D-isomer by C-6-deoxygenation of D-glucosamine (147, 148).

Hydrogenation of phenylhydrazones has been used by Wolfrom *et al.* (149) to synthesize several mono- and di-aminoalditols and a 5-amino-D-xylose derivative.

Another general method is the replacement of a tosyl group with ammonia (ammonolysis) or, better, by hydrazine (hydrazinolysis) followed by reduction. The reaction is carried out on tosyl groups isolated from adjacent free hydroxyls to prevent epoxide formation. The hydrazinolysis of 1,2-5,6-di-O-isopropylidene-3-O-D-glucofuranose has long been supposed to proceed without inversion, and a recent preparation of "3-amino-3-deoxy-D-glucose" by this method was described (150). However, Lemieux & Chu (151) found that the hydrazinolysis proceeds with inversion, yielding the 3-amino-3-deoxy-D-allose compound. Such inversion could be anticipated from related reactions reported by Cope & Shen (152). The structure of the amino allose was proved on shortening the carbon chain by removal of C-6 to form 3-amino-3-deoxy-D-ribose found in puromycin (see above). A similar shortening of the carbon chain of ethyl 2-acetamido-2-deoxy-1-thio-α-D-galactofuranoside led to 2-amino-2-deoxy-L-arabinose (L-arabinosamine) (153). Coxon & Hough (154) confirmed Lemieux & Chu's findings and proved the structure of the 3-amino allose by chain shortening at C-1, to yield 2-amino-2-deoxy-D-ribose (D-ribosamine). More examples of the inversion reaction have appeared in hydrazinolysis syntheses by Wolfrom *et al.* of D- and L-ribosamine, D- and L-lyxosamine, and 3,5-diamino-dideoxy-D-ribose (155). Hydrazinolysis at tosylated primary hydroxy groups, where inversion is ir-

relevant, was used to synthesize 5-amino-5-deoxy-D-xylose and 6-amino-6-deoxy-D-galactose (155). Cramer, Otterbach & Springmann (156) used ammonolysis, and also exchange of tosyl by azide followed by reduction, to synthesize methyl 6-amino-6-deoxy-α-D-glucoside, which proved difficult to hydrolyse. Repetition of the synthesis with a benzyl β-glucoside enabled the free sugar to be isolated, since benzyl is removable by reduction.

The ammonolysis reaction was used by Foster, Stacey & Vardheim (157) to synthesize 1,6-anhydro-D-altrosamine and by Jeanloz and co-workers (158, 159) to form 1,6-anhydro derivatives of D-gulosamine and 2,4-di-O-acetamido-2,4-dideoxy-D-glucose. Some of the 2-amino-aldohexoses, like the corresponding non-amino sugars, are prone to form 1,6-anhydro derivatives, and several of these are known (144).

Other synthetic methods of general utility are a ring-expansion method (furanose→pyranose), which allows the synthesis of the four possible methyl 3-amino-3-deoxy-D-xylosides (160), and a novel synthesis from a periodate-produced dialdehyde. In this latter synthesis, Baer & Fischer (161) oxidized methyl β-L-arabinopyranoside and treated the dialdehyde with nitromethane. Subsequent manipulation, as shown in Figure 5, gives, in theory, eight 3-amino-3-deoxy pentopyranosides, but it proved possible to crystallize the L-xylose derivative in good yield.

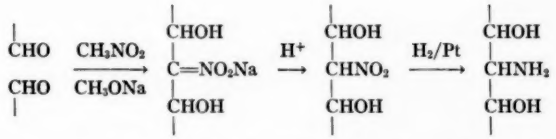


FIG. 5. Conversion of dialdehyde from methyl β-L-arabopyramoside into a methyl 3-amino-3-deoxypentopyranoside.

D-Galactosamine has been oxidized to D-galactosaminuronic acid (162) and D-glucosamine converted into its 6-sulphate (163). Synthetic chitosan sulphate has anticoagulant activity (164).

Miscellaneous.—Partial acid hydrolysis of chitosan followed by N-acetylation and fractionation yields pure N-acetyl 1→4-linked β-N-acetyl-D-glucosamine oligosaccharides (165). Substitution at C-3 of 2-amino sugars can be detected by the Elson-Morgan reaction (166). Crumpton (167) has described the following properties of all eight 2-amino-2-deoxy-D-aldohexoses: (*a*) displacement volumes of the hydrochlorides from ion-exchange resins, (*b*) R_F values of hydrochlorides and N-acetyl derivatives, and (*c*) paper-ionophoretic migration of the latter.

OLIGOSACCHARIDES

Reports of the isolation of new oligosaccharides continue, and the time for a compendium of the oligosaccharides, their properties, and derivatives is overdue. Wagner (168) has listed over 100 oligosaccharides. Reasons of

space restrict the following review mainly to derivatives of glucose and galactose.

Chemical syntheses.—The well-established methods of oligosaccharide synthesis are beginning to be directed towards polysaccharide synthesis. It is already possible to synthesize large molecules (degree of polymerization over 1000) by random polymerization (see Acid Reversion, pp. 111–13). Specific polymerization was achieved by Haq & Whelan (169) by self-condensation of 2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide with elimination of hydrogen bromide (the Koenigs-Knorr reaction), and 1→6-linked β-glucose polymers up to a nonasaccharide were prepared. The extension of this method is limited only by the preparation of suitable substrates. Another approach, by Husemann & Reinhardt (170), is the chemical modification of natural polymers, as in the addition of acetobromo-glucose, -maltose, and other brominated oligosaccharides to the primary hydroxyl groups of amylose.

The Koenigs-Knorr reaction, the main route for oligosaccharide synthesis, is limited by two factors. Firstly, the β-halogeno sugars are usually unstable, and, since condensation with acceptor sugars, with silver salts to take up the acid, is usually accompanied by Walden inversion, the synthesis of the α-linked oligosaccharides is not possible. Secondly, condensation at secondary hydroxyl groups is often slow and yields are poor. Recent developments may improve matters:

Mercuric cyanide as condensing agent led to the formation of both nigerose (α-1→3-diglucose) as well as laminaribiose (β-1→3-diglucose) in the reaction between α-acetobromoglucose and acceptors with unsubstituted C-3-hydroxyls [Matsuda & Sekiguchi (171, 172)].

A new method by Weygand, Ziemann, & Bestmann (173) for the preparation of glycosyl bromides caused the formation of a fairly stable form of β-acetobromoglucose. The reaction was the bromination of ethyl 1-thio-α-D-glucoside. The only stable β-halogeno glucose previously available, 3,4,6-tri-O-acetyl β-glucosyl chloride, has been used to synthesize nigerose (174) and kojibiose (α-1→2-diglucose) (175).

Curtis & Jones (176) have made the first direct synthesis of lactose and the excellent yield (35 per cent) was thought to be caused by the use of an acyclic compound (2,3–5,6-di-O-isopropylidene-D-glucose diethylacetal) as acceptor for α-acetobromogalactose.

An alternative approach to the formation of α-links in oligosaccharides may perhaps be made via the 2,4,6-trimethylbenzoyl β-glycosides described by Helferich and co-workers (177, 178). These react at room temperature with alcohols and an acid catalyst to form α-glycosides, and their attempted condensation with sugars would be a useful next step.

The acceptor molecule in the formation of 1→6-linked oligosaccharides is usually made by blocking the primary hydroxyl with trityl, esterifying the remaining hydroxyl groups, and then removing the trityl group. If silver perchlorate is used as condensing agent, the trityl-ester compound itself will condense, the displacement of trityl and addition of halogeno sugar occur-

ring in the same reaction sequence (179). Improvements in the preparation of sugar acceptors with free hydroxyls at C-1 are reported by Helferich & Steinpreis (180), who have synthesized several oligosaccharides with these acceptors.

Of potential use in oligosaccharide synthesis is the alkali stability of 1→2-linked aldoses. Thus, Kuhn, Baer & Gauhe (181) found that the glucose moiety of fucosido-lactose (2^{Gal}-α-L-fucosyl-lactose) is removed by alkali to give the 2-α-L-fucosyl derivatives of D-galactose (61 per cent) and D-talose (14.6 per cent), the latter a product of epimerization of the former. The 1→2-linked sugars are also distinctive in that they are almost non-reducing towards copper reagents (182).

Recent oligosaccharide syntheses by conventional Koenigs-Knorr methods are of 2-β-xylosyl-L-arabinose (183), 5-β-xylosyl-L-arabinose, 6-β-xylosyl-galactose (184), 3-β-galactosylgalactose (185), N-acetyl-lactosamine, 6-β-galactosylglucosamine (186), 6-β-glucosaminylglucosamine (187), and 6^1-β-glucosylmaltose (188).

Naturally occurring oligosaccharides.—Courtois (189) has reviewed the naturally occurring galactosylsucroses. Raffinose, stachyose, verbascose, and higher members of this 6^G-substituted sucrose series occur in broad bean (190), sage, and mullein (191). Courtois and co-workers (192) find that the roots of *Lychnis dioica* (red campion) contain two tetra-, two penta- and, two hexa-saccharides. These are 6^G, 1^F- or 6^G, 3^F-galactose-substituted sucroses. The former type (lychnose series) predominates in the autumn, while the penta- and hexa-saccharides of the latter type (isolychnose series) are obtained in June. All contain raffinose as part of their structures and can be named as follows: lychnose itself is 1^F-α-galactosylraffinose; isolychnose, the minor tetrasaccharide component, is 3^F-α-galactosylraffinose; the lychnose-type penta- and hexa-saccharides are 1^F-α-galactobiosyl- and 1^F-α-galactotriosyl-raffinose, respectively; the isolychnose penta- and hexa-saccharides are probably 3^F-α-galactobiosyl- and 3^F-α-galactotriosyl-raffinose, respectively.

Swietenose, extracted from gum mokha, is 6-α-galactosylgalactose (galactobiose) (193). Other galactose disaccharides, not occurring free, but as parts of polysaccharides, are 3- and 6-β-galactosylgalactose from golden apple gum (194), 3-α-galactosylgalactose and the corresponding trisaccharide from λ-carrageenin (195), and 5-β-galactofuranosylgalactose from galactocaralose (196).

Kojibiose (197), isomaltose, maltose, nigerose, maltulose, and turanose (198) occur in honey, maltulose and nigerose in beer (199), and laminaribiose and 3-β-cellobiosylglucose in the unfermentable residue of barley mash (200). The larvae of the nematode *Porrocaecum decipiens* from codfish muscle contain 6.0 per cent trehalose (dry weight) and 55 per cent glycogen (201). Trehalose also occurs in appreciable amounts in locusts where the body-fat tissue synthesizes it from glucose with ATP or UDPG or both (202).

The first unequivocal proof of the structures of isomaltose and gentiobiose has been obtained by Wolfrom, Thompson & Brownstein (68), and the

ring conformations of maltose and cellobiose have been defined by Bentley (203). The series of isomaltodextrins from the di- to the hexa-saccharide has been obtained by Turvey & Whelan after graded hydrolysis of dextran (204), and the sugars were used by Kabat (205) in defining the combining site in the dextran-antibody reaction. Gentiobiose occurs as part of the sugar moiety of the plant glycoside asiaticoside in which the sugar chain is 4^2-α-L-rhamnosylgentiobiose (206). A glucose hexasaccharide from placenta is claimed by Akiya & Tomoda (207) to be cellohexaose. This seems most unlikely, since the sugar acetate has $[\alpha]_D+168°$, as opposed to $-0.23°$ quoted for α-cellohexaose acetate (208).

Enzymic synthesis of oligosaccharides.—α-D-Galactosyl groups are transferred to D-mannose from phenyl α-galactoside by coffee bean α-galactosidase, forming 6-α-galactosylmannose (epimelibiose) [Courtois & Petek (209)], and from melibiose to C-6 of N-acetyl-D-glucosamine and -D-galactosamine by an extract of *Trichomonas foetus* [Watkins (210)]. Pazur and coworkers showed that β-D-galactosyl groups are transferred from lactose by *Saccharomyces fragilis* lactase to form 3-β- and 6-β-galactosylglucose and 6-β-galactosylgalactose (211), the acceptor sugars being made available by concomitant hydrolysis of lactose. 6-β-Galactosylglucose is further acted on to form 6-β-galactosyl-6-β-galactosylglucose (212).

Shukla & Prabhu (213) report that sucrose is synthesized from glucose-1-phosphate and fructose by a sugar cane enzyme. The pathway is, therefore, that found in *Pseudomonas saccharophilia* (214), rather than in wheat germ (215), where UDPG is the glucose donor. Barber (216) injected 2-deoxy-D-glucose into tobacco plants and isolated 6-β-fructosyl-2-deoxyglucose.

Bourne, Hartigan & Weigel (217) have investigated the reaction whereby dextransucrase preparations cause the transfer of glucose residues from sucrose to lactose or cellobiose to give "branched" trisaccharides. The products are 2^G-α-glucosyl-lactose and 2^1-α-glucosylcellobiose, respectively. The synthesis is, therefore, of α-1→2-linkages, rather than of the usual α-1→6-linkages synthesized by the enzyme, although it has to be proved that dextransucrase is the causative agent of this synthesis. The reaction is of interest as a possible route to branched polysaccharides. Formation of the lactose derivative followed by enzymic removal of the β-galactosyl group is a better route to kojibiose than is chemical synthesis (218).

Polymers that have not been prepared by chemical synthesis but are relatively easy to prepare with enzymes are the methyl α-glycosides of the maltodextrins, prepared from amylopectin and methyl α-glucoside with the potato transferase, D-enzyme, [Peat, Whelan & Jones (219)] and from Schardinger dextrins and methyl glucoside (α- and β-) by the action of *Bacillus macerans* amylase [Pazur, Marsh & Ando (220)]. Both enzymes can use a wide range of sugars as alternative acceptors, and the products are useful test substrates in studies of the specificity of other starch-metabolizing enzymes.

An *Alcaligenes* species forms novel compounds when grown in strongly

aerated cultures on lactose, lactobionate, maltose, or maltobionate [Bernaerts & de Ley (221)]. These are keto glycosides, produced by oxidation at C-3 of the non-reducing sugar unit, e.g., 4-β-(3-ketogalactosyl)-glucose from lactose.

Glucans

Starch.—New methods of fractionation and subfractionation of starch have revealed new features of the amylose (linear) and amylopectin (branched) components, linear and branched being used in a relative sense. It is now recognized that conventional methods of dissolving and fractionating starch can cause degradation. Gilbert (223) has discussed the two factors causing this degradation. One is hydrolysis; this may amount only to a few bonds per million, but the molecular weight of amylopectin is so large that this degree of hydrolysis causes a significant lowering in the size of the molecule. The second, and more important, factor is oxidative splitting of the chains, and it is now customary to carry out the dissolution of the starch under nitrogen (223), although some lowering of viscosity, possibly indicative of degradation, can still take place (222). Erlander & French (224) were able to disperse starch granules under helium in 6 *M* or 8 *M* lithium bromide without causing any detectable degradation. Baum & Gilbert (225) found that, at 15° under nitrogen, sodium hydroxide only dissolves amylose from potato starch. Montgomery & Senti (226) treated corn, wheat, and potato starches with various organic solvents and were then able fractionally to leach out the amylose at 98°, successive fractions having lower iodine stains and viscosities. The residue was a fairly pure amylopectin. Arbuckle & Greenwood and Cowie *et al.* (222, 227) found that aqueous leaching of oat and wheat starches at 70° removed a completely linear amylose fraction, representing about 40 per cent of the whole amylose. The test of linearity was complete hydrolysis by β-amylase (see below). The amylose remaining in the granule was of a higher degree of polymerization and was branched. Later work by Banks, Greenwood & Thomson (228) has shown that pretreatment of the granules with boiling 80 per cent methanol is essential for the success of this experiment. Amylose can also be subfractionated into fractions of differing degrees of polymerization by precipitation with iodine (229).

Perlin (230) subfractionated radioactive wheat starch by aqueous leaching and obtained two amylopectin components of differing solubility and differing specific activity. Other properties, such as iodine stain and average unit-chain length, were the same. The more soluble fraction had the same specific activity as the amylose component.

The incomplete degradation of amylose by pure β-amylase, first described by Peat, Pirt & Whelan (231), has been the subject of much investigation into the nature of Z-enzyme, which renders the amylose susceptible to complete β-amylolysis, and the nature of the barriers to the endwise action of β-amylase. The idea that Z-enzyme is a β-glucosidase (232) has been challenged (227), and the possibility that oxidation during the heating stages of amylose preparation can introduce barriers to endwise-acting enzymes, such

as β-amylase and phosphorylase, has been reviewed by Gilbert (223). Peat *et al.* (231) and Cowie *et al.* (227) found the degree of β-amylolysis of amylose to be increased on heating in alkali in air and concluded that barriers were not introduced. Gilbert (223) accepts that natural amylose contains an anomaly which impedes β-amylase but points out that experiments to test for the oxidative introduction of barriers are best performed with a linear amylose. Using a biosynthetic linear amylose, he found that heating in aqueous solution in oxygen lowered the degree of β-amylolysis. This has been confirmed by Banks, Greenwood & Thomson (233) who used the natural linear amylose fraction obtained by leaching starch granules (see above), and the results of comparable heating of natural potato amylose have been reinterpreted to show that barriers are indeed introduced. However, even when the product is prepared and recrystallized under nitrogen, the β-amylolysis limit of natural potato amylose is still only 77 to 85 per cent, according to the source (233). This apparently "natural" barrier to β-amylase is not, however, a barrier to potato phosphorylase [Baum, Gilbert & Scott (234)].

Studies in which starch was hydrolyzed in the presence of $H_2{}^{18}O$ show that both α- and β-amylase split the 1→4-glycosidic bonds between C-1 and the oxygen bridge (235, 236). The enzymes are, therefore, glycosylases rather than glycosidases. The present position as to whether β-amylase hydrolyzes one chain at a time (single-chain action), or all chains about equally (multi-chain action) is that natural amylose is degraded by single-chain action (237), but degradation of short chains (degree of polymerization of 50 or less) shows some of the characteristics of both types of action, the action pattern being temperature and pH dependant (238, 239).

Amylases which, like β-amylase, split starch by endwise action have been obtained in highly purified form from *Aspergillus niger* by Pazur & Ando (240) and as crystals from *Rhizopus delemar* and *A. oryzae* by Tsujisaka, Fukumoto & Yamamoto (241). The product is, however, glucose, not maltose, and the enzymes split the 1→6-branch points, which β-amylase does not do.

Very prolonged salivary α-amylolysis of starch was found by Ahnström (242) to yield 6^2-α-glucosylmaltose (panose) as the smallest molecule containing the original branch linkage. After successively less prolonged digestions, the smallest such products are a tetrasaccharide, 6^3-α-glucosylmaltotriose [Nordin & French (243)], and a pentasaccharide, 6^3-α-maltosylmaltotriose [Whelan & Bines (13)]. Pazur (244) has described the pattern of breakdown of maltodextrins by salivary α-amylase.

The synthesis of amylose other than from glucose-1-phosphate has been reported by Walker & Whelan (245), who incubated the maltodextrin-disproportionating D-enzyme with maltotetraose and, by continuously removing the glucose product with hexokinase and ATP, caused the equilibrium to be disturbed in favour of the synthesis of long linear chains. The reaction forms part of a scheme for the simultaneous synthesis of amylose and am-

ylopectin by a multi-enzyme system (246). Erlander (247) suggests that amylopectin is synthesized by a phosphorolytic debranching of glycogen. This scheme apparently has no place for Q-enzyme, which synthesizes amylopectin from amylose and which Peat, Turvey & Jones (248) have shown will not further increase the degree of branching of amylopectin to form glycogen. Studies by McConnell, Mitra & Perlin (249) on mature starch from wheat plants grown on [1-^{14}C]glucose or [1- or 2-^{14}C]acetate encourage the belief that amylopectin is synthesized from amylose and show that starch deposition in the kernel is not extensively reversible. Phosphorolysis of raw starch granules is reported by Turvey & Hughes (250).

French & Thoma (251) have separated each of the maltodextrins in the series from maltose to malto-octadecaose. These will be invaluable as model compounds. Dextrins of degree of polymerization of five and more enhance the tri-iodide ion spectrum; at eight or more, they give visible stains on paper with methanolic iodine and at 18 and above, give visible iodine stains in solution. French & Youngquist (252) find that the dextrins in the range 5 to 15 degree of polymerization form striking dichroic crystals in concentrated iodine, complex formation resulting from the inclusion of iodine by "interrupted" amylose helices. A crystalline maltodextrin (degree of polymerization 12 to 15) deposited from a sweet potato starch syrup could be obtained with A- or B-type x-ray patterns (253).

Glycogen.—As with the starch fractions, there has been extensive investigation of the possibility of degradation during the procedures commonly used for the isolation of glycogen. With one exception, there is now general agreement that the Pflüger method of extracting glycogen with hot 30 per cent potassium hydroxide causes degradation; this was shown by Stetten, Katzen & Stetten (254). Rabbit and rat muscle or liver glycogens extracted in this way have molecular weights from 2 to 6×10^6, while, if extracted with cold trichloroacetic acid, the molecular weight range is 11 to 80×10^6. The degradative effect of hot dilute (6 to 8 per cent) potassium hydroxide is even greater than of 30 per cent alkali and is more rapid in oxygen than in nitrogen (254, 255). Bryce, Greenwood & Jones (256) confirm that alkali degrades glycogen and suggest that the equal molecular weight of glycogen extracted by boiling water and that extracted, and degraded, by alkali (255) may be attributed to a preferential leaching of a lower molecular weight component by water. There is evidence of such lower molecular weight material in trichloroacetic-extracted glycogen (255), but degradation simply in hot water is also reported (257, 258). Cold water, however, was found by Orrell & Bueding (257) to extract glycogen from *Ascaris* muscle and rabbit liver having ten times the molecular weight of trichloroacetic acid-extracted glycogen and 50 to 100 times that of Pflüger glycogen. This cold water glycogen has a minimum molecular weight of 50 to 200×10^6, sediments at 5000 g, and is not an aggregate.

Holme, Laurent & Palmstierna (259) report different experiences with

glycogen extracted from *E. coli*, in that Pflüger glycogen has the higher molecular weight (40 to 90×10^6) and the trichloroacetic acid-extracted glycogen the lower.

Liddle & Manners (260) and Warren & Whittaker (261) have measured the average unit chain lengths and degrees of β-amylolysis and phosphorolysis of an extensive range of glycogens. The idea expressed by Peat *et al.* (262) that the debranching R-enzyme can be used to distinguish between amylopectin and glycogen, since steric effects prevent it from attacking the latter, must be modified in view of the finding by Fleming & Manners (263) that a rabbit liver glycogen of high average unit chain length (18 units) was attacked by R-enzyme. Gunja & Manners (264) have converted potato amylopectin into a glycogen-like polysaccharide by the action of the branching enzyme of brewers' yeast.

Walker & Whelan (265) have re-examined the structures of the rabbit muscle phosphorylase limit dextrins of glycogen and amylopectin. It is claimed that the Cori & Larner structure (266) is incorrect with respect to the lengths of the residual outer chains. These are now thought to be both four units long. The original postulates (266) were that the side chain was one unit long and the main chain about six units long.

Rosenfeld (267) studied the α-amylolysis of glycogen and found residual material of molecular weight 3 to 6×10^3 which was resistant to β-amylase and only slightly attacked on further α-amylolysis. Its content of 1→6-bonds was 33 to 47 per cent. It is not clear whether this is a polysaccharide distinct from glycogen or whether it is a part of the glycogen molecule in which the branch points are so close as to form a network impenetrable to α-amylase.

β-Glucans.—Laminarin, the food reserve of brown seaweeds (e.g., *Laminaria cloustoni*), was thought until recently to be simply a chain molecule of 1→3-linked β-D-glucopyranose units. Peat, Whelan & Lawley (83) found some of the chains to be terminated by glycosidic linkage with C-1 (or C-6) of D-mannitol, and β-1→6-links between glucose residues are also present. Goldstein, Smith & Unrau (268) find the mannitol to be di-substituted by glucose chains at C-1 and C-2 or C-5 and C-6. Electrophoresis in sodium hydroxide separates the mannitol-terminated chains (laminaritol, degree of polymerization 30) from the mannitol-free molecules (laminarose, degree of polymerization 17). A third carbohydrate component, mannose, has now been found by Smith & Unrau (269), and there is evidence from periodate oxidation studies, that some, at least, of the 1→6-links are not points of branching (270). Anderson *et al.* (53) have analysed laminarin by methylation and periodate oxidation and describe methods of estimating molecular size, mannitol content, and proportion of mannitol-terminated molecules. These measurements suggest that branched structures are present [see also (271)].

The β-1→3-glucosidic link also occurs in many other polysaccharides. It has recently been found in leucosin from the flagellated protazoon *Ochromonas mallamensis* (272), in callose from grape vine (273), and in pachyman

(274). This last polysaccharide constitutes more than 90 per cent of the dry weight of the fungus *Poria cocos* Wolf. Bakers' yeast glucan contains β-1→3- and β-1→6-glucosidic links in the ratio 10:1 (275) and oat β-glucan 1→4- and 1→3-links in the ratio 3:1 (45).

The full understanding of cellulose biosynthesis is brought nearer by Colvin's finding (276) that aqueous ethanolic extracts of *Acetobacter xylinum* contain a compound which is rapidly converted into cellulose when placed in water. This conversion is accelerated by a heat-labile extracellular substance from the culture medium, presumably an enzyme. Neither UDPG nor short β-glucose chains are the immediate precursors of the cellulose. A new crystalline modification of cellulose is reported (277).

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GLYCOLIPIDS[1]

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This review will be concerned only with those complex lipids that contain carbohydrates in combination with long-chain aliphatic acids or alcohols. Derivatives of glycerol containing no other carbohydrate are not considered, and sterol glycosides are excluded. Portions of the review are devoted to discussions of older literature concerning compounds not previously mentioned in lipid reviews of this series, since no previous discussion has been limited exclusively to glycolipids. While no claim of completeness is intended, an attempt has been made either to discuss or to provide reference to all carbohydrate-containing lipids that have been investigated within the past decade.

Reviews of glycolipids have been prepared by Lauenstein (1) and Lederer (2). Several other reviews have covered parts of this field, usually in the course of discussion of a related field, such as those of Klenk (3), LeBaron (4), Folch & LeBaron (5), Carter (6), Carter *et al.* (7, 8), and Westphal (9). Radin has discussed chromatography (10) and has provided a fine chapter on analysis of glycolipids (11).

Glycolipids frequently combine the polar features of polyols with the lipophilic behavior of extended aliphatic chains. Thus, some glycolipids, while showing considerable solubility in "lipid solvents" such as chloroform or chloroform-methanol mixtures, will also form aqueous solutions. The combination of polar and non-polar properties makes attractive the speculation that glycolipids may be structural features of lipid-aqueous interfaces.

Solutions of glycolipids in water seem to consist of aggregations of molecules that show a high molecular weight when examined in the ultracentrifuge. Some investigators have taken this characteristic as an indication that the materials are high molecular weight polymers, while others have considered it to be merely an example of micelle formation. Some glycolipids, such as the bacterial lipopolysaccharides, are undoubtedly polymeric, since a polysaccharide can be split from the lipid portion, but definite information on the situation with the mucolipids of nervous tissue and red blood cells is lacking.

Carbohydrate Esters and Glycosides

Corn smut glycolipids.—Several years ago, Haskins (12) reported the formation of a crystalline glycolipid produced extracellularly when corn smut (*Ustilago zeae*) was grown in aerated submerged cultures. The chemistry of

[1] The survey of the literature pertaining to this review was completed in October, 1959.

this substance was investigated by Lemieux *et al.* (13, 14). The material, which could be obtained in yields of 3 to 4.5 gm./l. by extraction of the culture solids with methanol, proved to be a mixture of closely related acids. Since this mixture of "ustilagic acids" could not be resolved, a study of degradation products was undertaken. The facile saponification in dilute alkali at room temperature (sap. eq. 383 to 392), followed by acidification, steam distillation, and extraction, gave acetic, caproic, 3-hydroxyhexanoic, and 3-hydroxyoctanoic acids, along with an amorphous mixture of "glucoustilic acids." The latter compounds, which provide the structural framework of the intact molecules, were shown to be β-D-cellobiosides of two palmitic acid derivatives, 15-D,16-dihydroxyhexadecanoic acid and 2-D,15-D,16-trihydroxyhexadecanoic acid (15, 16). The β-hydroxy acids have the L-configuration (17). On the basis of their studies of the degradation products, Lemieux *et al.* (18) propose that the ustilagic acid mixture consists of a monoacetyl-mono-3-L-hydroxyhexanoyl substituted di-β-D-glucosyl-15-D,16-dihydroxyhexadecanoic acid, a monoacetyl-mono-3-L-hydroxyoctanoyl substituted di-β-D-glucosyl-2-D,15-D,16-trihydroxyhexadecanoic acid, and, possibly, a third component containing caproic acid. The position of substitution of the acyl groups and of the cellobiose unit was not investigated.

Haskins & Thorn (19) have demonstrated that the ustilagic acids are responsible for the antibiotic activity of *Ustilago* cultures. These workers have also studied optimum conditions for the production of ustilagic acids (20) and the biosynthesis of these materials (21). Glucose-1-^{14}C is incorporated directly into the glucose of ustilagic acid, as well as undergoing some randomization to the C-6 labeled compound, presumably through three carbon units. The fatty acids seem to arise by the normal condensation of two carbon compounds.

Boothroyd, Thorn & Haskins (22, 23) have isolated another interesting glycolipid from the culture medium of an *Ustilago* species (PRL 627). This material occurs as an impure oil of high specific gravity, 1.035 at 25°, and in yields of 15 gm. per liter of culture medium. It is a non-reducing material consisting only of fatty acids and carbohydrate. Alkaline saponification (sap. eq. 245 to 312), followed by acidification and steam distillation, gave acetic acid, while methanolysis gave methyl esters of saturated and unsaturated acids from C_6 to C_{18}. The water-soluble fraction yielded a methyl glycoside of D-mannose and *meso*-erythritol. Since crystalline D-manno pyranosyl-1-*meso*-erythritol had been isolated from the culture medium in good yield, it was assumed that the glycolipid was an acylated derivative of this carbohydrate.

Rhamnolipid.—A crystalline acidic glycolipid which is formed in the culture medium of *Pseudomonas aeruginosa* was isolated and characterized several years ago by Jarvis & Johnson (24). This compound was obtained in high yields (2.5 gm./l.) only when the organism was grown on a glycerol-containing medium. Unlike the ustilagic acids, this material appeared to be homogeneous, giving, on hydrolysis, equimolar quantities of L-rhamnose

and a levorotatory 3-hydroxydecanoic acid. The acid was later shown to be of the D series by Lemieux & Giguere (17) and was prepared synthetically by Serck-Hanssen & Stenhagen (25). The molecular weight of the rhamnolipid, determined by the neutral equivalent (665) and the Rast method (660), indicated that two moles of each component were present.

The compound was non-reducing, indicating that the sugar molecules were glycosidically bound. On hydrolysis, a fragment that consisted of two moles of 3-hydroxydecanoic acid in ester linkage could be isolated. Finally, the intact compound consumed two moles of periodic acid. These data indicated the structure shown in Figure 1 for rhamnolipid:

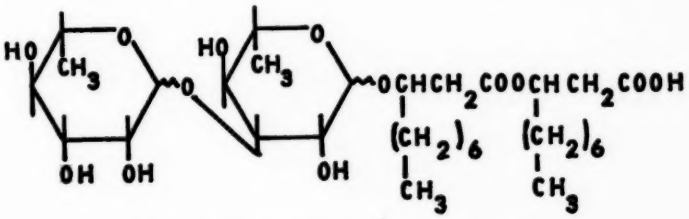


FIG. 1. Rhamnolipid from *Pseudomonas aeruginosa*.

Rhamnolipid is probably closely related to the *Pseudomonas* metabolites investigated earlier by Bergström, Theorell & Davide (26). Rhamnolipid shows bacteriostatic activity toward *Mycobacterium tuberculosis*, strain H37Rv, and is toxic to mice.

Hauser & Karnovsky (27, 28) have carried out extensive investigations on the biosynthesis of rhamnolipid. They have shown that labeled acetate or glycerol is readily incorporated into the 3-hydroxydecanoic acid in a fashion consistent with a two-carbon condensation mechanism. α-^{14}C-Glycerol labels carbons 1, 3, 4, and 6 of rhamnose equally, while β-^{14}C-glycerol labels carbons 2 and 5 equally. Therefore, rhamnose probably arises in this organism by condensation of three carbon units readily derivable from glycerol. In this regard, it is of interest that Hauser & Karnovsky (29) have shown that rhamnolipid is also formed from DL-propanediol-1,2. Growth of the organism in the presence of DL-1-^{14}C-glycerol and various concentrations of propanediol gave rise to rhamnose in which the specific activity was very nearly the same as the specific activity of carbon in the medium, and both halves of the molecule were essentially equally labeled. The authors interpret these findings as indicative of a common intermediate readily derivable from both glycerol and propanediol-1,2.

Cord factor.—An interesting glycolipid, which may be extracted from virulent strains of *Mycobacterium tuberculosis* by means of hydrocarbon solvents, has been termed "cord factor," because it seems to be associated with the characteristic tendency of these organisms to aggregate in long cords

(30). Extraction of the material does not kill the bacilli, but it decreases their virulence. Bloch & Noll (31) have discussed the biological properties of this material. It shows a marked toxicity in mouse tests, being most effective when injected in repeated small doses.

The chemistry of cord factor has been investigated by Noll & Bloch (32) and Asselineau & Lederer (33). Earlier studies established that the material was an ester of a large aliphatic acid, 3,x-dihydroxymycolic acid[2] and glucose. The isolation of trehalose from hydrolysates of cord factor and methylation and periodate studies indicated that the molecule is the 6,6′-dimycolate of trehalose (34), as illustrated in Figure 2. This structure has been confirmed by synthesis (35).

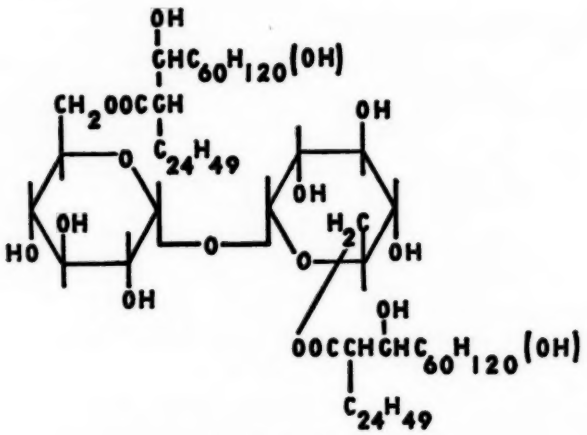


FIG. 2. Cord factor.

Lederer (2) has discussed the toxicity of synthetic carbohydrate esters. The 6-mycolates of several hexoses are toxic (37) but do not aggravate tuberculosis infections as does cord factor. Lower esters of trehalose, such as the 6,6′-didocosanate, as well as the 6-monomycolate and the 2,6,6′-trimycolate, have greatly reduced toxicity.

The lipids of *Corynebacterium* species, which are toxic, have been shown to contain carbohydrate esters (38). Lederer *et al.* (39, 40) have isolated a fatty acid, corynemycolic acid ($C_{32}H_{64}O_3$), from these organisms. Although no natural derivatives of this material have been isolated, Diara & Pudles (41) have synthesized the 6,6′-dicorynemycolate of trehalose. This compound, however, was found to be non-toxic.

[2] Several mycolic acids have been isolated and characterized from various strains of *Mycobacterium* [see Asselineau & Lederer (36)]. All are very large aliphatic acids. The position of the second hydroxyl group in the cord factor mycolic acid is unknown, as indicated in Figure 2.

It is of some interest that several glycolipids, consisting only of fatty acids in combination with carbohydrates, show marked biological activity. No information is available on the mechanism of toxicity of these materials for microorganisms and higher animals.

Ascarosides.—Fouquey, Polonsky & Lederer (42, 43) have investigated a group of glycolipids that constitute the waxy non-saponifiable material from the eggs of parasitic worms (*Parascaris equorum*). This material had been named "ascarylic alcohol" by earlier investigators.

Chromatography on alumina resolved ascarylic alcohol into three components, called ascarosides A, B, and C, in order of elution. All were shown to be glycosides of higher secondary fatty alcohols combined with a 3,6-dideoxyhexose, ascarylose. Thus, ascaroside A contains a normal secondary alcohol, $C_{28}H_{58}O \pm CH_2$, while ascarosides B and C contain a diol, 2,6-dihydroxyhentriacontane, $C_{31}H_{64}O_2$. Ascaroside B contains one mole of ascarylose, and ascaroside C contains two, with one molecule of sugar glycosidically bound to each hydroxyl group. Ascarylose is a non-crystalline oil which can be distilled at low pressure. Subsequent investigation (44 to 46) has shown ascarylose to be 3,6-dideoxy-L-arabinohexose. It is the optical antipode of tyvelose, an immunologically active fragment from the lipopolysaccharide endotoxin of Gram-negative bacteria. These substances will be discussed later.

The ascarosides may exist as esters of acetic and propionic acid in the unfertilized eggs of parasitic worms (42).

Glycerol Glycosides

Lipids containing galactosyl glycerols.—Carter, McCluer & Slifer (47) have isolated, from a benzene extract of bleached wheat flour, two glycolipid fractions that contain D-galactose and glycerol. When hydrolyzed, each fraction yielded a mixture of galactosyl glycerols. Periodate oxidation and enzymatic cleavage studies established that these were β-D-galactosyl-1-glycerol and α-D-galactosyl-(1$\rightarrow$6)-β-D-galactosyl-1-glycerol. Similar carbohydrates had been isolated previously from plant sources (48, 49).

Zentner (50) has shown the presence of galactosyl glycerol lipids in wheat gluten, and Daniels (51) has found them in untreated flour. Mason & Johnston (52) have reported a careful investigation of the lipids from unbleached flour milled from two different types of wheat. The lipids were extracted with water-saturated butanol-1, which the authors claim to be a superior solvent compared to ethanol-ether mixtures. The acetone insoluble lipids were subjected to extensive countercurrent distribution that yielded fractions containing galactosyl glycerides. The major portion of the glycolipids were digalactosyl glycerides, with only traces of monogalactosyl glycerides. The authors postulate that chlorine bleaching may degrade the digalactosyl glycerides to the monogalactosyl glycerides found by Carter *et al.* (47). The fatty acids from the glycolipids were separated by gas chromatography and shown to consist of 15 per cent C-16 acids and 85 per cent C-18 acids.

Recently, Benson *et al.* (53, 54) have shown that glycolipids become

labeled quickly when *Chlorella* suspensions are exposed to $^{14}CO_2$ and light. The lipids were identified by deacylation and paper chromatography of the resulting water-soluble compounds. The compounds that were found included a monogalactosyl monoglyceride, a digalactosyl monoglyceride, and a β-D-galactosyl-6-sulfate monoglyceride. This was the first report of the existence of a sulfatide of this type.

Glycerol Phosphatides

Phosphatidyl inositol.—Klenk (3) has commented on the ubiquitous occurrence of inositol phosphatides and has reviewed the evidence that indicates a phosphatidyl inositol structure for the most common member of this group. Recently, the stereochemistry of this compound has been elucidated by the careful investigations of Pizer & Ballou (55). A few comments on the chemistry of *myo*-inositol and its phosphate esters are necessary for a discussion of this work.

Myo-inositol is generally considered to exist in a chair-form ring in which five hydroxyls have an equatorial disposition and one hydroxyl has an axial

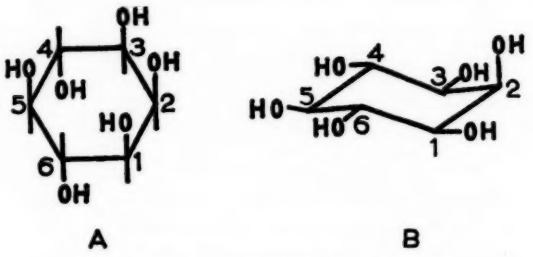


Fig. 3. Two representations of *myo*-inositol.

disposition, as shown in Figure 3B. Stereochemistry and nomenclature in this series have been discussed by Lardy (56) and Magasanik & Chargaff (57).

On the basis of examination of models, Magasanik & Chargaff (57) expressed the opinion that the hydroxyl groups of this compound are unfavorably located for the formation of cyclic five-membered rings such as the isopropylidine derivatives. Hawthorne & Chargaff (58), on the basis of analogy, considered the formation of cyclic phosphate esters to be unlikely in the inositol series. This assumption was cast into doubt by the fact that phosphatidyl inositol, when hydrolyzed, gives both glycerol phosphate and inositol phosphate, the latter presumably being formed by way of a cyclic ester (see Figure 4) (59, 60).

This anomaly is clarified in the discussion of Angyal & Macdonald (61), who point out that, if *myo*-inositol is presumed to exist in a chair-form ring, then the distance between any vicinal hydroxyl oxygen atoms is identical (2.86 A), and that this distance is too great for a one-carbon bridge. However,

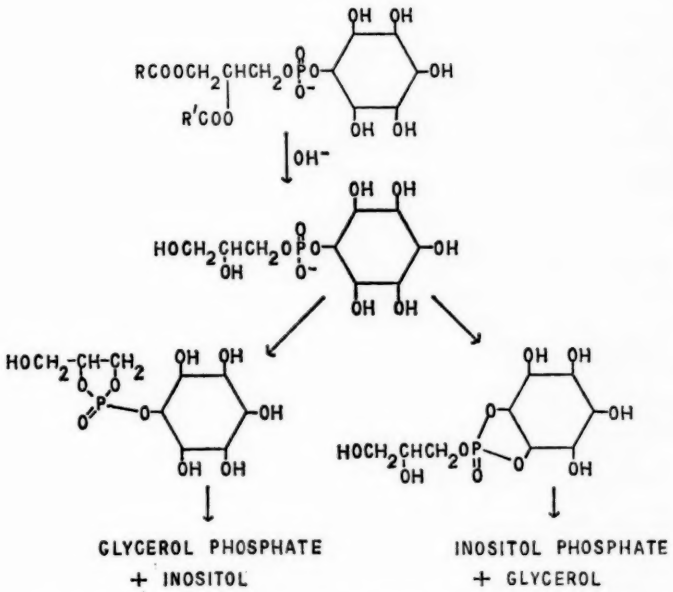


FIG. 4. Interpretation of the alkaline hydrolysis of phosphatidyl inositol which leads to both glycerol phosphate and inositol phosphate.

if two *cis*-hydroxyls (e.g., 1 & 2 or 2 & 3) are brought closer together, the ring becomes less puckered and the distance between other axial substituents is increased; thus, this process requires little expenditure of energy. Conversely, two *trans*-hydroxyls cannot be brought closer together without great expenditure of energy. These authors demonstrate their point by preparation of the 1,2-isopropylidine *myo*-inositol.

The extension of this argument to the case of cyclic phosphate derivatives finds confirmation in the synthesis of the cyclic 1,2 *myo*-inositol phosphate by Posternak (62) and by Pizer & Ballou (55).

A phosphate ester devoid of optical activity and identical with the synthetic *myo*-inositol-2-phosphate prepared by Iselin (63) has been obtained by the partial hydrolysis of inositol hexaphosphate (phytin) by alkali or by enzymes. This point has been discussed and amply confirmed by Brown *et al.* (64 to 66), Kilgour & Ballou (67), and Pizer & Ballou (55). Hawthorne (68) obtained an optically inactive inositol phosphate by acid hydrolysis of inositol phosphatides, and he assumed this to be identical with the authentic 2-phosphate. This has been subjected to more careful examination in the recent work of Pizer & Ballou (55). They have demonstrated, by means of a paper

chromatography system that separates the isomeric *myo*-inositol phosphates, that acid hydrolysis leads to an optically inactive mixture of the ±1-phosphate and the 2-phosphate.

More enlightening is the finding of Pizer & Ballou (55) that alkaline hydrolysis of phosphatidyl inositol yields an ester differing from that obtained from phytin, particularly in the fact that it is optically active. This must, therefore, be either a 1- or 4-phosphate ester.[3] A small amount of the 2-phos-

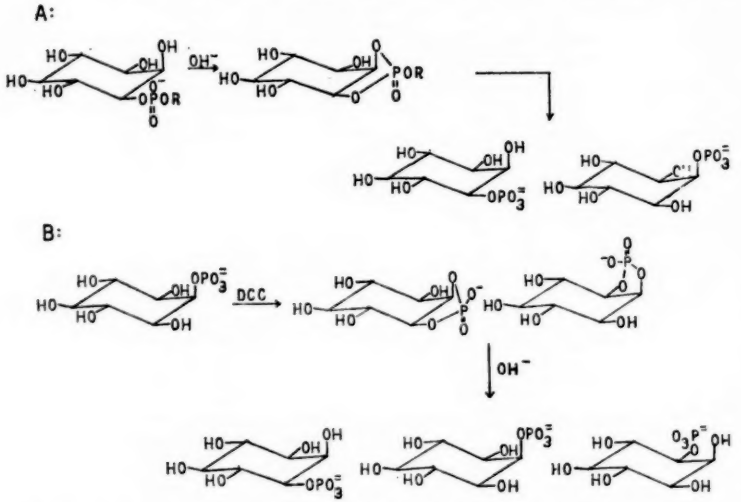


FIG. 5. A. Stereochemical course of alkaline hydrolysis of a phosphodiester containing *myo*-inositol. The asymmetric *myo*-inositol-1-phosphate ester leads to the 1,2-phosphate which cleaves to a mixture of the optically active 1-phosphate and the optically inactive 2-phosphate.

B. Forced cyclization of *myo*-inositol-2-phosphate with dicyclohexylcarbodiimide leads to a racemic mixture of 1,2-phosphates, which are cleaved in base to give the optically inactive 2-phosphate and a racemic mixture of the enantiomorphic 1-phosphates.

phate ester was also formed during alkaline hydrolysis. The interpretation of this result is that the 1,2-cyclic phosphate ester is formed in the course of hydrolysis and that this material is opened in base to give a predominance of the 1-phosphate and some 2-phosphate (see Figure 5A). The fact that an optically active product was obtained indicates that the original attachment of phosphate was to the asymmetric 1-carbon, since the authors have shown that forced cyclic phosphate formation by treatment of the 2-phosphate ester with dicyclohexylcarbodiimide followed by alkaline hydrolysis leads to

[3] The 3- or 6-phosphates would be enantiomorphs of these [see Lardy (56)].

an optically inactive mixture of the 2-phosphate and the ±1-phosphate (see Figure 5B).

Confirmatory evidence is provided by Brockerhoff & Hanahan (69), who subjected phosphatidyl inositol to mild basic hydrolysis and obtained glycerol phosphoryl inositol, which was degraded by a selective periodate oxidation procedure. This destroyed the glycerol portion of the molecule while leaving the inositol intact. The resulting product was optically active, and since the asymmetry caused by glycerol had been destroyed, this property could only be attributed to attachment of the phosphate at an asymmetric carbon of inositol.

Finally, Ballou & Pizer (70) have recently succeeded in synthesizing an optically active *myo*-inositol 1-phosphate. Starting with galactinol (1-O-α-D-galactopyranosyl *myo*-inositol), they obtained 2,3,4,5,6-penta-O-benzyl *myo*-inositol. This was phosphorylated and debenzylated to give a *myo*-inositol 1-phosphate with an optical rotation equal to, and in the opposite direction from, that shown by the natural ester from soybean phosphatides. This compound is, therefore, the enantiomorph of the phosphate ester from the soybean lipid. Since the stereochemistry of galactinol is known (71), the structure of the natural phosphatidyl inositol can be represented as shown below.

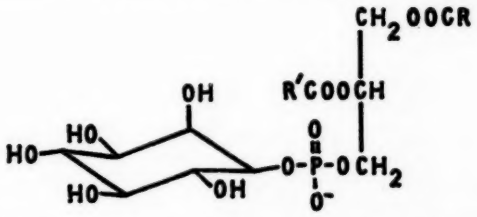


FIG. 6. Probable stereochemistry of phosphatidyl inositol.

Using the nomenclature proposed by Lardy (56), the soybean lipid yields L-*myo*-inositol 1-phosphate. The argument of Hawthorne & Hübscher (72) in favor of the 2-phosphate is based upon the fallacious assumption that the phosphate ester formed by alkaline hydrolysis of phosphatidyl inositol is optically inactive.

Davies & Malkin (73) have reported the synthesis of a phosphatidyl inositol containing *myo*-inositol 2-phosphate. This was accomplished by reacting glycerol 1-iodide-2,3-distearate with the silver salt of 2-(1,3,4,5,6 pentacetyl) *myo*-inosityl phenylphosphate, followed by deacylation and removal of the phenyl protecting group. The product (free acid) was a crystalline solid with a melting point of 135–137°C.

Studies by Agranoff, Bradley & Brady (74) indicated that, unlike the biosynthesis of other glycerol phosphatides [see Kennedy (75, 76)], phosphatidyl inositol was formed by the reaction of a diglyceride derivative with free inositol. All cytidine nucleotides stimulated the incorporation of tritium-labeled

inositol into the phospholipids of guinea pig kidney. When tritium-labeled cytidine monophosphate was incubated in this cell-free, particulate system, a chloroform-methanol-soluble radioactive product was formed which could be degraded by hydrolysis to radioactive cytidine monophosphate. It was, therefore, postulated that a lipid-soluble cytidine nucleotide, possibly cytidine diphosphate diglyceride, was involved in this reaction.

Paulus & Kennedy (77, 78) have studied a system of guinea pig liver microsomes that synthesizes much larger quantities of inositol phosphatide than the system studied by Agranoff *et al.* (74). They believe the latter system involves an exchange reaction rather than a *de novo* synthesis. The incorporation of labeled inositol into the liver microsome system is not stimulated by cytidine nucleotides, but incorporation of ^{32}P from α-glycerol phosphate-^{32}P is stimulated by cytidine triphosphate and free inositol; other cytidine nucleotides are inactive.

Paulus & Kennedy (79), using a chicken liver microsome system, have confirmed the formation of an ether-soluble nucleotide when cytidine diphosphate-^{32}P and DL-α-glycerophosphate are incubated together. This nucleotide does not accumulate in the presence of inositol. These authors have synthesized a cytidine diphosphate dipalmitin that reacts with ^{3}H-inositol to give radioactive lipid and cytidine monophosphate. Thus, the sequence of reactions is:

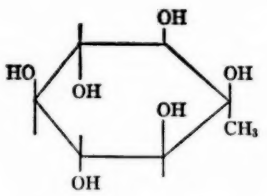
$$\text{CTP} + \text{L-}\alpha\text{-glycerophosphate} + 2\text{RCOSCoA} \rightarrow \rightarrow \text{CDP diglyceride} \qquad 1.$$

$$\text{CDP diglyceride} + \text{inositol} \rightarrow \text{phosphatidyl inositol} + \text{CMP} \qquad 2.$$

Equation 1 represents a number of reactions in which the sequence of steps is not known.

The enzyme system studied by Thompson, Strickland & Rossiter (80) seems to be more complex than the kidney and liver systems. The presence of an energy-producing glycolytic system and the possibility that more than one inositide is formed probably contribute to the difficulties encountered in this brain homogenate system.

Posternak *et al.* (81, 82) have studied the incorporation of an inositol antagonist, isomytilitol:



into the phospholipid of *Neurospora crassa* and of a *Schizosaccharomyces*. This material became incorporated into lipids as a phosphate ester.

Hokin & Hokin (83 to 85) have studied what appears to be an exchange reaction occurring in inositol phosphatides and phosphatidic acid during

secretory processes. Using $^{32}PO_4$, ^{3}H-inositol, and glycerol-1-^{14}C, they have found that the turnover of two inositides in pigeon pancreas slices is greatly increased during acetylcholine stimulation of protein secretion. In inositide A all three substances are turned over, but in inositide B glycerol incorporation is not stimulated. These two inositides seem to be very similar in composition, but are readily separated by paper chromatography. In this regard, it should be noted that Marinetti, Erbland & Stotz (86), using chromatography, have observed good separation of two ionic forms of phosphatidyl serine; caution in the interpretation of chromatographic separation of ionic lipids should be observed.

Hokin & Hokin (87) propose that the phospholipids take an active part in the secretory mechanism. They suggest that complexes of the lipids with proteins facilitate transport across the lipid boundaries of cells and that hydrolysis of the lipid portions at the outside aqueous interface frees the protein into the surrounding medium. Regeneration of the phosphatides would explain the observed turnover of labeled substrates.

Dawson (88 to 90) has found that an inositol phosphatide and a phosphatidyl glycerol (cardiolipin) activate phospholipase B of *Penicillium notatum*. A similar activation of cabbage phospholipase D has been noted by Weiss *et al.* (91). Bangham & Dawson (92) have found that the activating lipids cause a marked change in the charge on the lecithin molecule at pH 3.3 and that they render the lecithin non-extractable with ether (90). They postulate that the activation process is an effect on the lecithin surface rather than on the enzyme molecule. It should be noted that rather large amounts of activator are required, usually 10 to 20 per cent of the weight of substrate present. This might be explained by Dawson's observation that an enzyme present in the *Pencillium notatum* preparation hydrolyzes phosphatidyl inositol (93).

Another interesting case of activation of enzymes by phosphatidyl inositol is supplied by Ambe & Venkataraman (94). They report that a purified soluble form of cytochrome oxidase is inactive in catalyzing the oxidation of reduced cytochrome-*c* by oxygen unless supplemented with a phospholipid and that phosphatidyl inositol is most active in this capacity.

Huggins *et al.* (95, 96) have investigated a phosphatidopeptide fraction from renal cortex. After rather drastic treatment of the tissue with heat and solvents, the phosphatidopeptide fraction may be extracted with acidic solvents. The material bears a resemblance to the phosphatidopeptide of Folch & LeBaron (97) except that it yields a monophosphoinositide rather than a diphosphoinositide, as does the compound from brain [see LeBaron (4)]. The mixture also contains sphingosine in low concentration along with fatty acids and hexose.

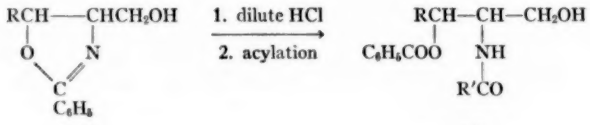
More complex derivatives of phosphatidyl inositol have been investigated. Vilkas & Lederer (98) reported the presence of a phosphatide from a streptomycin-resistant strain of *Mycobacterium tuberculosis* H37Rv, which contained, in addition to a phosphatidyl inositol structure, two moles of D-mannose. Vilkas (99) has shown that this compound is a diacyl glycerophos-

phoryl inositol dimannoside. Evidence for a phosphatide containing inositol and five moles of glucose was also reported. Existence of polymannosides among the lipids of *M. tuberculosis* was investigated by Pangborn (100) and Nojima (101, 102). From a mixture of polymannosides, Nojima (103) has isolated a degradation product that has an analysis consistent with a barium salt of glycerophosphoryl inositol pentamannoside. The fatty acids combined with the oligomannosides seem to be palmitic and tuberculostearic acid (10-methyl stearic).

Complex plant phosphatides containing inositol will be discussed later.

Glycolipids Containing Long-Chain Bases

Cerebrosides.—The chemistry of cerebrosides has been reviewed by Carter Galanos & Fujino (7). During the past year, Shapiro & Flowers (104) have reported the synthesis of a dihydrocerebroside. The reaction sequence used was:

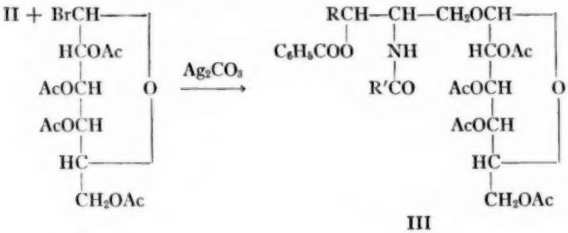


3.

I II

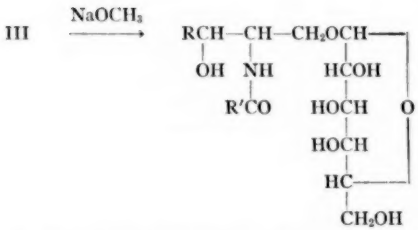
R = $CH_3(CH_2)_{14}$; R' = R, or $CH_3(CH_2)_{16}$

Compound II has the *erythro* configuration about carbons 2 and 3 of the base.



4.

III



5.

Radin & Kishimoto (105) and Skipski *et al.* (106) have demonstrated that the α-hydroxy acids from cerebrosides are a mixture of homologues of which

the C-18 and C-24 compounds predominate. Sweeley & Moscatelli (107)[4] have investigated and developed a new technique for studying long-chain bases. The bases are freed by hydrolysis and then subjected to oxidative cleavage by periodic acid. The resulting aldehydes are separated by gas chromatography. These workers have shown that most long-chain bases are mixtures of closely related species. Thus, sphingosine mixtures contain sphingosine, dihydrosphingosine, and, probably, dehydrosphingosine. It is apparent that the sphingolipids are inhomogeneous with regard to both the aliphatic base and the fatty acids. This explains the difficulty of obtaining pure sphingolipids that show well defined crystals and give satisfactory analytical data.

Rosenberg & Chargaff (108) have reinvestigated the cerebroside content of the spleen of Gaucher patients. Trams & Brady (109) have found that, although ^{14}C-labeled sugars were incorporated into the cerebroside of Gaucher spleen, tritium-labeled sphingosine was not. Details of preparation of the tritium-labeled sphingosine were not given, though it should be pointed out that, if the Wilzbach technique (110) were used, it is likely that dihydrosphingosine would be the labeled product, rather than sphingosine, since Dutton *et al.* (111, 112) have shown that oleic acid is reduced to stearic acid under these conditions.

Earlier studies on the incorporation of various labeled substrates into cerebrosides were discussed by LeBaron (4). A recent paper by Moser & Karnovsky (113) contains further details on results reported earlier.

Interest in cerebroside sulfuric acid, the structure of which was elucidated by Schmidt *et al.* (114), has centered on its formation in tissue (115 to 117).

Lees *et al.* (118, 119) have reported that a sulfatide fraction which contains phosphorous can be separated from cerebroside sulfate by solvent fractionation techniques. However, this fraction can be further resolved by chromatography on Florisil into a phospholipid and a material that appears to be merely cerebroside sulfate. Jatzkewitz (120) has also reported the presence of a second sulfatide containing phosphate from the brain tissue of leucodystrophy patients. This sulfatide was well separated from cerebroside sulfate by paper chromatography. Preparative scale paper chromatography gave sufficient material for analysis, which showed the presence of fatty acid, sphingosine, galactose, sulfate, and phosphate. In the light of the data of Lees *et al.* (119), it would seem that caution should be exercised in interpreting these results, and the pitfall of separating ionic forms of lipids (86) should be considered.

Fujino & Negishi (121) have reported the occurrence of an abalone enzyme that cleaves the sulfate from cerebroside sulfate. Higher homologues of cerebrosides that contain disaccharides or oligosaccharides have been investigated. Rapport *et al.* (122, 123) have studied a material, which they have termed "Cytolipin H," obtained from human epidermoid carcinoma grown

[4] The author is grateful to Dr. C. C. Sweeley for making available a manuscript of his publication prior to its appearance in the journal.

in cortisonized rats. This material is quite similar to the cerebroside obtained by Klenk & Rennkamp (124) from ox spleen, although it shows minor differences in physical properties (125), and seems to be a cerebronylsphingosylglucosyl galactose. Of particular interest is the fact that, when this cerebroside in minute concentration (1×10^{-8} μg.) is mixed with lecithin and cholesterol, it fixes complement with the rabbit anti-human reticulum-cell sarcoma serum.

No new material on the chemistry of the complex lipids of the brain (ganglioside, mucolipid, strandin) or of the blood cell lipids (hematoside, globoside) has appeared since the reviews of Klenk (3) and LeBaron (4). Several workers (113, 126, 127) have demonstrated the incorporation of labeled compounds into these substances.

A very interesting report on the relationship between complex lipids and tetanus toxin has been made by van Heyningen (128, 129). This work was initiated as a reinvestigation of a 1906 report by Landsteiner & Botteri (130) that phrenosine served as the receptor of tetanus toxin. This was anomalous since the toxin is fixed more readily in gray matter than in white matter, while phrenosine is more plentiful in white matter. Van Heyningen found that the protogon fraction (complex lipids) fixed tetanus toxin readily and that, at high ionic strength, no other protein was fixed. The resulting insoluble complex could be centrifuged and analyzed for protein, thus providing a convenient assay of toxin receptor. When the mixture of complex lipids was fractionated by countercurrent distribution, it was found that two fractions were necessary for activity in this assay. A complex containing equal weights of cerebroside and ganglioside (or strandin) with calcium ion functioned very well as an activator. However, this complex seems only to confer insolubility on the toxin-ganglioside compound, since it was shown that ganglioside alone could fix tetanus toxin.

Complex plant glycolipids.—Complex glycolipids of plant seeds have been the subject of many past investigations. The work of Hawthorne & Chargaff (58), Hawthorne (68), Woolley (131), Folch & Woolley (132), Folch (133), Malkin & Poole (134), and Scholfield *et al.* (135) indicated that complex materials containing glycerol, phosphate, inositol, fatty acids, nitrogenous bases, and sugars were present. However, these reports were inconsistent as to the relative amounts of these constituents and their mode of linkage. Carter *et al.* (136) have investigated a sphingolipid obtained by mild saponification of a crude plant seed phosphatide mixture. This material, termed phytoglycolipid, is a family of closely related lipids that contain fatty acid, phytosphingosine, phosphate, inositol, a hexuronic acid, D-glucosamine, arabinose, galactose, and mannose. Degradation of the material gave phytosphingosine, N-cerebronylphytosphingosine, N-cerebronylphytosphingosyl-1-phosphate, inositol phosphate, an oligosaccharide, and an acidic, non-reducing trisaccharide containing only inositol, D-glucosamine, and D-glucuronic acid (137). Recent studies show that mannose and glucuronic acid are linked to inositol, glucosamine is joined to glucuronic acid, and galactose and arabinose are bound to

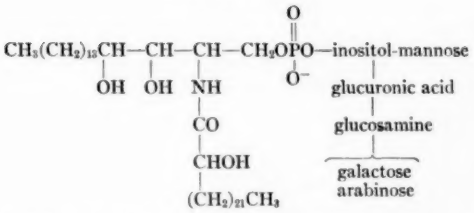


FIG. 7. Partial structural representation of phytoglycolipid.

glucosamine. Thus, nitrite causes deamination of the glucosamine portion to give a compound containing inositol, mannose, and glucuronic acid, as well as a chitose, arabinose, and galactose portion.[5] A partial structural formula is shown in Figure 7.

Phytoglycolipid may be an artifact of the alkaline saponification employed for isolation of the material, and the native lipids may be more complex (137). Hörhammer *et al.* (138) have recently demonstrated the separation of a complex inositide from commercial soybean lecithin on paper impregnated with formaldehyde polymers.

Phytosphingosine was first reported as a component of the non-saponifiable fraction of yeast lipids (139) and was assigned a C-20 structure. The material found by Carter *et al.* (140) in plant seeds is a C-18 compound. Several other investigators have studied this problem [O'Connell & Tsien (141); Proštenik & Stanačev (142); Oda (143)], but the recent data of Sweeley & Moscatelli (107) is definitive on this point. Using the sensitive method of periodate cleavage followed by gas chromatography of the resulting aldehydes, these workers have obtained evidence that the plant seed lipids contain saturated and unsaturated C-18 bases. Since both a C-16 and a C-15 aldehyde were obtained, they conclude that some dihydrosphingosine is present in plant seed lipids. The yeast lipids contain both a C-18 and a C-20 base.

The presence of dihydrosphingosine in plant lipids would permit the speculation that phytosphingosine might arise from dihydrosphingosine by a hydroxylation reaction. If such a sequence exists in plants, it might also exist in animals, since phytosphingosine could give rise to sphingosine by dehydration. Such a scheme would be similar to that proposed by Bloomfield & Bloch (144) for the desaturation of fatty acids. However, evidence that the conversion of dihydrosphingosine to sphingosine is a dehydrogenation reaction is offered by Brady & Koval (145), who observed reduction of Safranin T when dihydrosphingosine was incubated with a particulate system of rat brain. This evidence is hardly conclusive, and this point would bear further investigation.

Oda & Kamiya (146) have isolated an N-acyl phytosphingosyl-1-phosphate from autolyzed yeast cells. This material is also a degradation product

[5] Dr. Herbert E. Carter, personal communication.

of phytoglycolipid (137). Thus, the intact yeast sphingolipid may be similar to phytoglycolipid.

Bacterial lipopolysaccharides.—Westphal & Lüderitz (147) have summarized the chemical information of the lipopolysaccharides of Gram-negative bacteria in 1954, and Westphal *et al.* have discussed this material more recently (9, 148). Since that time, considerable further information has become available on the chemical and biological properties of these materials. The lipopolysaccharides are protein- and nucleic acid-free preparations obtained by phenol extraction of the organisms. They may be cleaved by treatment with strong acid into a degraded polysaccharide and a phospholipid, lipid A.

The polysaccharide contains several interesting carbohydrates, including a heptose, fucosamine, rhamnose, and dideoxyhexoses (147, 149). The latter components are characteristic of particular organisms and have the structures shown in Figure 8. The structures of abequose, tyvelose, and paratose have been confirmed by synthesis (44, 46, 150).

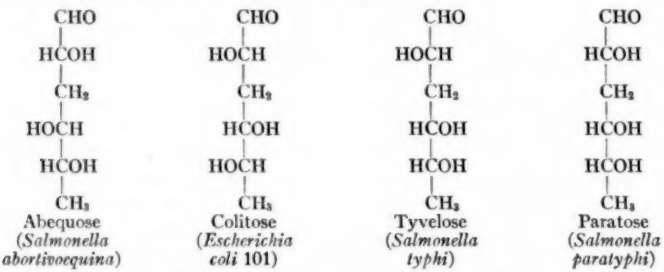


FIG. 8. Structures and sources of the dideoxyhexoses.

The dideoxyhexoses are immunologically active, inhibiting the precipitation of bacterial polysaccharide (antigen) by means of an antiserum prepared against this antigen (149). This property offers a convenient and sensitive method for differentiating the compounds.

The chemistry of lipid A was investigated by Ikawa *et al.* (151, 152). This material contains the novel long-chain base necrosamine (4,5-diaminoeicosane), as well as 3-hydroxymyristic acid, lauric, myristic, and palmitic acids, D-glucosamine, phosphate, ethanolamine, and aspartic acid.

Nowotny *et al.* (153) have reported that hydrolysis of intact lipopolysaccharides releases a series of fatty acids having from 12 to 24 carbon atoms. Lipopolysaccharides from several bacterial species gave the same spectrum of fatty acids.

The lipopolysaccharides have the remarkable physiological property of inducing fever in mammals when injected in minute doses (9, 148). This behavior seems to be associated with the lipid A portion, for, although the lipid itself is not very active, it becomes so when it is allowed to complex with a protein (casein) before injection.

Lipids of Great Complexity and of Uncertain Structure

Asselineau *et al.* (154) have isolated, from the "D waxes" of strains of *M. tuberculosis*, complex glycolipids which contain, in addition to sugars and fatty acids, several amino acids. The glycolipid from the Brévannes strain, for example, has a molecular weight of about 54,000 and consists of about 50 per cent mycolic acid. The remainder of the molecule is composed of L-arabinose, D-galactose, and D-mannose, amino sugars, alanine, glutamic acid, and diaminopimelic acid. Partial degradation of the material gave a peptide containing alanine, diaminopimelic acid, and amino sugar. This fact, with the observation that the carbohydrate-peptide portion is stable to base, indicates that the amino acids are joined to carbohydrate through a peptide bond.

Collins (155, 156, 157) has isolated a glycolipid derivative from sheep brain lipids. The lipids were first treated with 2,4-dinitro-1-fluorobenzene and diazomethane, and the products were fractionated by solvent partition and countercurrent distribution. The purified material contains glycerol, glucose, choline, ethanolamine, serine, fatty acids, a fatty alcohol, and other unidentified materials. On hydrolysis, the material is reported to give glycerol-1-phosphate, an unknown nitrogen-containing material, and a water-soluble material with an absorption maximum in the ultraviolet at 257 mμ. Collins has also isolated the fatty alcohol $C_{30}H_{50}O_4$ (mol. wt. 470 to 477) and the ultraviolet-absorbing material from lipids not exposed to dinitrofluorobenzene and diazomethane. It should be noted that treatment with diazomethane has been observed to be detrimental to some phospholipids (158, 159).

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THE STRUCTURE OF PROTEINS[1]

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In his Lane Lectures (1), the late Professor Linderstrøm-Lang advanced the suggestion that the structure of a protein could, most conveniently, be examined at three different levels which he called the primary, secondary, and tertiary structures. By "primary", he meant the sequence of the amino acid residues in the peptide chains, whereas the "secondary" structure describes the way in which the polypeptide chains are folded, and the "tertiary" how the folded polypeptide chains are arranged in space and are packed into the protein molecules. Here, cross-linkages, such as disulfide bonds, and interactions between the side chains of the amino acid residues enter. How much this terminology has influenced the thinking of protein chemists became particularly apparent during the four afternoon sessions of the Fourth International Congress of Biochemistry in Vienna where problems relating to the structure, biological activity, and biogenesis of proteins were discussed (2). It is, therefore, felt that tribute should be placed here to the man who has held such a unique place in the development of protein chemistry.

At present, there appears to be no major obstacle in the quantitative determination of the composition and amino acid sequence of proteins, and one may be tempted to predict a quick succession in the establishment of the primary structure of a great number of proteins. However, as was also pointed out by Hill, Kimmel & Smith (3), a word of caution with regard to such optimism should be inserted. The higher the molecular weight and the greater the number of peptide chains in a protein, the greater the number of hitherto unknown problems that will confront the investigator engaged in sequence work.

Since last years's chapter on "The Structure of Proteins" (3) dealt in detail with the procedures used and the problems encountered in the investigations of primary structure, this review will be confined to certain aspects of the secondary and tertiary structure. However, a brief discussion of the reactions that involve the peptide bond per se will be included.

There is justification for limiting the scope of this review. We have now reached the point where methods have become available for the study of the secondary and tertiary structure, and a detailed comparison can, therefore, be made between some of the results obtained and certain features that emerge from the sequence work or from other chemical properties. The reader should particularly be referred to the work on pancreatic ribonuclease. In view of the recently completed elucidation of its amino acid sequence (4), this protein may well be suited for establishing the relationship

[1] The survey of the literature pertaining to this review was concluded in November, 1959.

between primary, secondary, and tertiary structure and biological activity. A few examples where such attempts are now being made will be given.

PRIMARY STRUCTURE

Reactions Involving the Peptide Bond

The classification of the elements of protein structure as primary, secondary, and tertiary presupposes that the polypeptide chain in a given molecule is normal (5). Although little evidence exists for modifications of the peptide chain that involve the backbone of proteins, one cannot exclude the possible existence of structures such as oxazolines and thiazolines, as suggested by Bergmann & Miekeley (6) and Linderstrøm-Lang & Jacobsen (7). In such cases, five-membered rings would be formed by a reaction of the side chains of serine and of cysteine with the enolized peptide group

Oxazolines are, at least theoretically, possible intermediates in the acyl shift (8, 9), which has recently been reported by Edman & Josefsson (10 to 14) to occur in lysozyme and ribonuclease, as shown in Figure 1. This reac-

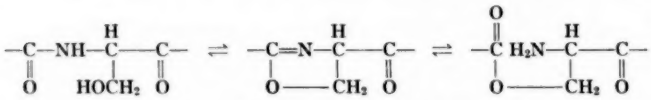

Fig. 1. Oxazolines and the Acyl Shift.

tion takes place in anhydrous formic acid and supposedly involves the hydroxyamino acids, serine and threonine. Edman & Josefsson found that during the formic acid treatment the enzymes are inactivated and the electrophoretic mobility, as compared with that of the native enzymes, is increased. These investigators ascribed the increase in mobility to the formation of new amino groups. Moreover, the enzymic activity could be restored in aqueous solvents of pH 7.0 to 8.0, and it was suggested that the reactivation coincided with a shift back to the peptide form.

This line of investigation has recently been extended by Smillie & Neurath who found that trypsin is also inactivated if treated with anhydrous formic acid for 14 to 17 hr. (15). In contrast to the observations of Edman & Josefsson, Smillie & Neurath were unable to detect the appearance of new amino groups in the inactivated trypsin by three independent methods: the ninhydrin color, the reaction with dinitrofluorobenzene, and electrophoresis. Titration data indicated that, if these groups exist, they cannot have a pK of less than 10.5. The results obtained by these investigators, therefore, do not support the hypothesis of an N- to O- acyl shift in trypsin produced by anhydrous formic acid, and they suggest two other mechanisms: the reaction proceeded only to the intermediate hydroxyoxalodine structure suggested by

Elliott (9), or acylation of the hydroxyl groups of the serine and threonine residues takes place.

Narita simultaneously concluded that formic acid transforms the aliphatic hydroxyls of serine and threonine into formate esters and does not cause a significant N-O-peptidyl shift (16). Narita followed the extent of formylation of bovine serum albumin, β-lactoglobulin, ovalbumin, lysozyme, O-acetyl-DL-serine, N-acetylserine, and O-acetylseryltyrosine by use of radioactive formic acid as a tracer.

In a recent study, Fasman has investigated the N-O-acyl shift in poly-DL-serine. With the aid of infrared spectroscopy, he was able to demonstrate that an N-O shift occurs if poly-DL-serine is treated with concentrated sulfuric acid. However, under the experimental conditions chosen, only 70 per cent of the original amide bonds rearranged, whereas the remaining hydroxyls of the serine residues were sulfonated (17). In view of the somewhat conflicting results obtained by the different groups, further work is needed to define the conditions under which acyl shifts occur in proteins or polypeptides.

A different line of investigation is that of Bock & Thakur (18), who treated glycylserine, glycylthreonine, insulin, ribonuclease, glucagon, serum albumin, and lysozyme with anhydrous sulfuric acid to bring about conversion to ester bonds of those peptide bonds adjacent to the hydroxyamino acids. On subsequent treatment with 2 *N* salt-free hydroxylamine of pH 7.3, the esters were hydrolyzed. The stoichiometry and specificity could then be determined by: (*a*) colorimetric determination of the hydroxamic acid formed; (*b*) periodate determination of the serine groups exposed; (*c*) fluorodinitrobenzene determination of N-terminal groups produced; and (*d*) chromatography of the cleavage products. This method of Bock & Thakur appears to be promising and may be of value in locating the serine residues in proteins.

Another interesting type of reaction involving the peptide bond has been studied by Witkop and co-workers. These investigators applied the concept of selective activation of the inert peptide groups adjacent to an aromatic amino acid residue by making the peptide bond participate in intramolecular displacement reactions (19 to 22). Thus, Witkop and collaborators succeeded in cleaving the C-tryptophyl bonds in a number of proteins by the use of N-bromosuccinimide. They were able to demonstrate cleavage by measuring the release of new N-terminal residues. Bond cleavages generally averaged 20 to 40 per cent. The number of new N-terminals formed corresponded to the number of tryptophan residues in the molecule. The results of Witkop *et al.* indicate the presence of Try.Lys and Try.Ala bonds in tobacco mosaic virus protein, a Try.Ala bond in human serum albumin, Try.Gly and Try.Ser in bovine serum albumin, and Try.Phe and Try.Arg in lysozyme.

Finally, if lysozyme is treated with methanol or ethanol, using trifluoroboride as catalyst, a preferential cleavage occurs — restricted to peptide bonds in which glycine, alanine, and aspartic acid enter [Edman & Josefsson (23)]. The reaction, however, is biphasic and only during the initial fast phase has

specificity been observed. Prolonged exposure to the reagents, particularly at temperatures above 20°C., leads to hydrolysis of the protein.

SECONDARY STRUCTURE

The Conformations of Polypeptide Chains

So much has been written about the α-helix in proteins (24) and on the relative stability of the helix as compared with the random coil (25, 26) that we shall refer the reader to the literature for further details. Similarly, the different inter- and intramolecular forces responsible for maintaining the native configuration of proteins have been adequately discussed (27 to 29). In experimental studies, however, work has been confined to an examination of a few properties that reflect directly the conformation of the peptide chain itself; only these properties will be considered here.

Optical Rotatory Properties

Polypeptides.—During the past few years, extensive research on the optical rotatory properties of polypeptides has been initiated in several laboratories. Since the results of these investigations have influenced considerably the interpretation of the optical rotatory behavior of proteins, they will be mentioned here briefly. Detailed information on this subject can be found in the chapter on "Polypeptides and Proteins" by Blout (30).

Yang & Doty (31, 32), Doty *et al.* (33), and Moffitt & Yang (34) observed that, in weak hydrogen bond-breaking reagents, the helical forms of poly-γ-benzyl-L-glutamate and poly-L-glutamic acid show a complex rotatory dispersion, whereas, in solvents in which the polypeptides have a random coil conformation, the dispersion is simple and follows the one-term Drude equation:

$$[\alpha]_\lambda = \frac{K'}{\lambda^2 - \lambda_c^2} \qquad 1.$$

Although considerable advance has been made in the theoretical analysis of the rotatory properties of helical molecules which describe adequately the behavior of some polypeptides (31, 35, 36, 37), the theory for the variations encountered in proteins is undoubtedly more complicated. The formulae derived by Moffitt, Fitts & Kirkwood (38), while of theoretical interest, cannot be applied to the experimental data. They have led, however, to the development of equation 2, which is of value in describing the optical rotatory

$$[\alpha]_\lambda = \frac{100}{M} \cdot \frac{n^2 + 2}{3} \left[\frac{a_0 \lambda_0^2}{(\lambda^2 - \lambda_0^2)} + \frac{b_0 \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \right] \qquad 2.$$

properties of certain polypeptides and, as will be shown below, of fibrous proteins. Constants characteristic for the helix are b_0 and λ_0, whereas the a_0 term contains both the helix and the amino acid residue contribution to the specific optical rotation. In solvents in which the polyamino acids, e.g., poly-L-glutamate, poly-γ-benzyl-L-glutamate, poly-L-alanine, poly-L-leucine, or poly-L-lysine, are present as fully coiled, right-handed α-helices, the value of

b_0 is -600 to -650, whereas, for the random coil forms, b_0 becomes 0 and $\lambda_0 = 210$ mμ (cf. 30).

Fibrous proteins.—Cohen & Szent-Györgyi (39), in 1957, investigated the rotatory properties of a number of fibrous proteins with an α-type, wide-angle x-ray diffraction pattern. These proteins show a complex rotatory dispersion which can be fitted to Equation 2. The investigators pointed out that the coefficient b_0 of Equation 2 could be used as a measure of helix content. Using poly-γ-benzyl-L-glutamate as a model, they assigned a value of -630 to b_0 to a fully coiled, right-handed α-helix. Thus, in the denatured state, $\lambda_0 = 210$ mμ, and b_0 becomes 0, as was previously found for polypeptides (cf. 30).

Table I lists the results of Cohen & Szent-Györgyi and the measurements recently reported by Kay & Bailey on *Pinna nobilis* tropomyosin (40). Kay's study of this protein (41), which has a molecular weight of 130,000 (light-scattering and sedimentation-diffusion), indicated it to be a rigid rod-like molecule with the dimensions of an α-helix (1400 A long and 18.5 A wide) and confirms the conclusions drawn from the rotatory properties.

Szent-Györgyi & Cohen (42) have further pointed to a possible inverse relationship between the amount of proline and hydroxyproline in proteins and their α-helix content (Table II). According to these investigators, proteins that, in the native state, are more than half helical contain less than 3 per cent proline (42). In view of this suggestion it will be of interest to investigate the rotatory behavior of proteins with an intermediate content of proline, let us say 3 to 7 per cent (43).

Collagen and gelatin.—In contrast to the fibrous proteins, the optical rotatory properties of native collagen obey a single-term Drude equation (44). The λ_c of collagen and gelatin is 205 ± 15 mμ and changes little during denaturation or, as shown by Harrington, in the presence of strong salt solu-

TABLE I

OPTICAL ROTATORY PROPERTIES OF MUSCLE PROTEINS IN NATIVE AND DENATURED STATE*

Protein	$-[\alpha]_D$ nat.	b_0	$-[\alpha]_D$ den.	λ_0
				(mμ)
Light Meromyosin I	13.0	$-660°$	118	212
Tropomyosin	16.0	$-620°$	118	213
Paramyosin	11.1	$-600°$	63	—
Light Meromyosin	20.4	$-490°$	107	215
Myosin	28.7	$-370°$	108	218
Heavy Meromyosin	34.5	$-300°$	103	215
Pinna nobilis Tropomyosin	11.7‡	$-650°$	109†‡	210†

* Cohen & Szent-Györgyi (39); Kay & Bailey (40)

† 8.0 *M* urea was used as denaturing agent, but 9.5 *M* urea in the other cases.

‡ $[\alpha]_{5890}$

TABLE II

HELIX CONTENT AND PROLINE CONCENTRATION OF MUSCLE PROTEINS*

Protein	Weight Per Cent Helix	Weight Per Cent Proline	Number of Non-Helical Residues per Proline Residue
Light Meromyosin I	100	0.22	—
Tropomyosin	94	0.35	15
Paramyosin	91	0.21	36
Light Meromyosin	74	0.97	23
Myosin	56	2.08	18
Heavy Meromyosin	45	2.87	16

* Szent-Györgyi & Cohen (42).

tions, such as lithium bromide, sodium bromide, and sodium thiocyanate (45). On the other hand, the changes of the specific optical rotations, $[\alpha]$, on denaturation are large. Thus, collagen resembles in its behavior the polyamino acid, poly-L-proline. Although no theoretical interpretation of the optical rotatory behavior of collagen can be attempted at present, one has to keep in mind that this protein has a high content of proline and hydroxyproline. The five-membered pyrrolidine ring must, therefore, impose stereochemical conditions not encountered with other fibrous proteins. A study of the rotatory behavior of poly-L-proline (46 to 48) and of proline-glycine copolymers (46) will, therefore, be of importance in assessing the collagen system.

Globular proteins.—From the studies prior to 1957 on optical rotation of globular proteins, three generalizations emerged: (*a*) the rotatory dispersion of most globular proteins in solution obeys a one-term Drude equation (44, 49, 50); (*b*) the value of the specific rotation, $[\alpha]_D$, of native proteins ranges from $-30°$ to $-70°$ and becomes 20° to 60° more negative upon denaturation (51 to 53); and (*c*) the dispersion constant, λ_c, of globular proteins is in the range of 230 to 270 mμ but is lowered to 220 mμ on denaturation (50).

During the past two years, Jirgensons has investigated the optical rotatory properties of a number of globular proteins and has arrived at a classification into three groups (54 to 57): (*a*) proteins the λ_c of which decreases from values above 240 mμ to values below 240 mμ upon denaturation (serum albumins, metal-binding β-globulins, taka-amylase); (*b*) those in which λ_c is in the intermediate range and varies little on denaturation (ovalbumin, chymotrypsinogen, chymotrypsin, γ-globulin, α-casein, ribonuclease, and lysozyme); (*c*) proteins with a low dispersion constant in the native form, i.e., in the range of "denatured" proteins of group (*a*), in which λ_c equals 200 to 220 mμ (Bence-Jones protein, pancreatic amylase, pepsin, trypsin inhibitor, rennin). Moreover, in group (*c*), λ_c increases upon denaturation. In addition, Schellman showed that the dispersion constant of small basic proteins, such as clupein, is low, i.e., $\lambda_c = 209$ mμ (58). Glucagon and fetuin, in-

vestigated by Kay & Marsh (59), belong to group *a*. A summary of these results, as well as of those of Schellman, is given in Table III.

A word of caution is necessary with regard to the use of the "λ_c-method" for estimating the percentage of helical structure in a protein (32). A low λ_c does not necessarily reflect the complete absence of α-helices. The shortness of helices, the simultaneous existence in the protein of right-handed and left-handed helical segments, or a different folding may account for the low values of λ_c. That changes in the levorotation of $[\alpha]_D$ always reflect helix-coil

TABLE III

OPTICAL ROTATORY PROPERTIES OF SOME PROTEINS IN THE NATIVE AND DENATURED STATE

Protein	Native			Denatured		
	$-[\alpha]_D$	λ_c	Solvent	$-[\alpha]_D$	λ_c	Solvent
Ovalbumin	28	266	H_2O	98	226	8.6 *M* urea
β-Lactoglobulin	28	245	0.1 *M* KCl	117	225	8.5 *M* urea
Insulin	29	266	H_2O	88	226	9 *M* urea
Lysozyme	50	257	H_2O	—	—	—
Bovine Serum Albumin	59	265	H_2O	109	221	8 *M* urea
Fetuin	55	260	0.2 *M* phosphate	79	225	8 *M* urea
Ribonuclease	74	233	H_2O	109	220	8 *M* urea
α-Chymotrypsinogen	66	241	H_2O	112	220	8 *M* urea
Chymotrypsinogen	78	239	H_2O	117	228	8 *M* urea
Glucagon	44	231	0.1 *M* glycine	63	222	8 *M* urea
Pepsin	63	216	0.1 *M* acetate	63	217	8 *M* urea
Rennin	—	210	0.25 *M* NaBr	—	—	—

transitions should also be questioned in view of the findings of Markus & Karush (60) and Turner *et al.* (61), who noted a decrease in the levorotation of serum albumin upon reduction or oxidation of the disulfide bonds. Whether the presence of the intact —S—S— bridges in serum albumin limits the amount of helical structure, which on rupture of the disulfide cross linkages is somewhat enhanced (60), or whether the increase of $[\alpha]_D$ can be ascribed solely to the high levorotation of cystine (61) cannot be decided without some independent estimates of the helix content by other methods.

Effect of solvents on the conformation of proteins.—In 1957, Harrington & Schellman suggested that the addition to a protein solution of lithium bromide, a reagent known to alter the activity of water, should result in helix formation. With oxidized ribonuclease, $[\alpha]_D$ of -91.1 increased to $-58.5°$ and λ_c rose from 225 mμ to 249 mμ when the salt concentration was raised to 7.0 *M* (62). A similar observation was made with silk fibroin (62). In contrast, the rotatory dispersion constant, λ_c, of serum albumin (62),

gelatin (45), and pepsin (43) did not change upon addition of lithium bromide; thus, ambiguity arises as to how this salt affects the conformation of a protein (cf. also 63, 64). A second solvent to which hydrogen bond-strengthening activity has been ascribed is 2-chloroethanol (32). Doty observed in several proteins changes of the optical rotatory properties which he ascribed to an increase in their helix content (65). In an extension of this work, Tanford & Weber have shown that in 2-chloroethanol the transition of native ribonuclease to the highly "helical" form occurs in two stages through an intermediate which has about the same optical rotatory characteristics as the native protein but which has a higher intrinsic viscosity. These investigators suggest that native ribonuclease in aqueous solvents is compactly folded, partly because of hydrophobic bonds, but that in 2- chloroethanol additional intramolecular hydrogen bonds are formed (66).

Another interesting application of the optical rotation method to the study of protein structure emerges from an investigation of Kay & Marsh (67) who followed the optical rotatory changes associated with the dimerization of bovine mercaptalbumin with mercurials. These investigators observed a small increase in the levorotation in the wave length range of 365 mμ to 577 mμ during the first 30 min. of the dimerization reaction. On the other hand, if dissociation of the dimer to the monomer is followed polarimetrically, the levorotation decreases to the original value of the monomer. On the basis of the large positive entropy which accompanies the dimerization reaction (68, 69), Edsall and co-workers had suggested that a slight unfolding occurs in the immediate vicinity of the sulfhydryl group involved in the dimerization reaction (70). Although the increase in the levorotation is not sufficiently large to state unequivocally that it represents a "true" conformational change, the experimental evidence, however, suggests that an unfolding does occur.

Another line of investigation is that of Markus & Karush (71), and Winkler & Markus (72), who have reported that the optical rotatory dispersion of various anionic azo dye-serum albumin complexes, outside of the region of the dye absorption maximum, differs considerably from that of the protein without added dye. Since dyes have no optical activity, these investigators interpreted the differences to be the result of structural changes that occur in the serum albumin molecule upon binding of the dye. A more plausible explanation, however, emerges from the recent study of Blout & Stryer (73) who observed that if the dyes rhodamine, at pH 4.6, or acriflavin, at pH 4.9, are added to the polyamino acid, poly-α-L-glutamic acid, a complex is formed which shows marked anomalous dispersion in the wave length range that corresponds to the absorption bands of the dye. Thus, the results of Blout & Stryer demonstrate clearly that, in the interpretation of the optical rotatory dispersion, one must take into consideration that if an optically inactive molecule is bound to an asymmetric polymer it may contribute considerably to the optical rotatory properties by creating a new asymmetry within the dye-polymer complex; without that a conformational change takes place.

Although a critical survey of the optical rotation measurements has been given by Kauzmann (26) and Schellman & Schellman (74, 75), we should like to stress once again that our interpretation of the optical rotatory properties of proteins at present is based on the polypeptide poly-L-glutamate as a model and on the proposal of Yang & Doty that the rotatory behavior of this polyamino acid, i.e., the α-helix and the randomly coiled form, suffices to explain the characteristics of proteins. These investigators calculated from the value of b_0 (Equation 2) the percentage of peptide bonds involved in the helical conformation and assigned a λ_c of 212 mμ to a protein in randomly coiled form. A higher value of λ_c, therefore, should indicate that a certain fraction of the polypeptide chain is folded in a helical structure (32). Although, in the case of ribonuclease and ovalbumin, a helical conformation of 15 per cent and 40 per cent, respectively, has been estimated and is supported by independent experimental evidence, some of the examples given in Table III indicate that the λ_c method cannot be used in all cases.

How diversified the problem actually is emerges from recent work in the polypeptide field. Blout & Karlson (76) and the Courtauld group (77) reported that the optical rotatory behavior of poly-β-benzyl-L-aspartate indicated the presence of a helix of opposite twist. Moreover, Fasman & Blout (78) showed that a different type of fold exists in poly-L-serine and an anomalous rotatory behavior has also been found for poly-L-tyrosine (79 to 81), ascribed to side chain interaction, and for poly-L-histidine (82). It is quite clear that the work on polyamino acids is of great importance for the interpretation of the optical characteristics of all high molecular weight substances. Undoubtedly, it will guide protein chemists in assessing the different properties encountered in proteins and in elucidating their secondary and tertiary structure.

Relation of Volume Changes to Chain Conformation

A hitherto little explored property is that of volume changes observed during denaturation. Kauzmann, with the aid of dilatometry, has found that the volume contraction that occurs on titration of ovalbumin to pH 2.0 is that to be expected from electrostriction of the water as the charge on the protein is increased. With bovine serum albumin, however, the volume contraction is large and is accompanied by an increase in the levorotation. Addition of 0.15 *M* potassium chloride eliminates the change in $[\alpha]_D$, but restores only half of the volume contraction (83). Kauzmann suggested that dilatometric measurements in conjunction with the study of the optical rotatory behavior may add an independent method for investigations concerned with the conformation of the peptide chain of proteins.

Hydrogen-Deuterium Exchange

The rate of exchange of hydrogen atoms with deuterium atoms provides a valuable tool in the study of the secondary structure of proteins. Lenormant & Blout were the first to show that, unless serum albumin, ovalbumin, and γ-globulin are denatured by heat or alkali, some of the hydrogen atoms

of these proteins can exchange slowly with deuterium (84). This type of experimentation has been extended by Linderstrøm-Lang and collaborators by use of a sensitive density method (85, 86). From the kinetics and the extent of exchange, the Carlsberg group demonstrated that the rate of exchange is increased at higher pH values or in the presence of urea and guanidine hydrochloride; moreover, it is temperature-dependent. From these observations and the early ones of Lenormant & Blout, it became apparent that the hydrogen atoms within those portions of the molecule that are stabilized by a helical structure, exchange more slowly than those in chain segments of random coil conformation. The deuterium-exchange method, therefore, provides an independent method for the estimation of the percentage of helical conformation in a protein. In the case of insulin (85), ribonuclease (87), and whale myoglobin (88), a comparison has been made of the amount of helix derived from deuterium exchange with that obtained from x-ray diffraction and optical rotatory dispersion measurements, respectively, and is given in Table IV. Considering the different principles involved in these three methods, the agreement is quite satisfactory.

TABLE IV

COMPARISON OF HELIX CONTENT OF PROTEINS OBTAINED BY DIFFERENT METHODS

Protein	Helix Content (in Per Cent)		
	Deuterium Exchange	Rotatory Dispersion	X-ray Diffraction
Ribonuclease	41	20*	—
Myoglobin	51	—	70
Insulin	48	38*	50

* Estimated from λ_c.

As previously discussed, a slow exchange of some hydrogen atoms has been observed in insulin, ribonuclease (85, 89, 90), and β-lactoglobulin (91). Although a rapid exchange should be expected with the isolated B-chain of insulin, Leach & Scheraga found that the rate was slow but somewhat faster than that found for the intact protein. The B-chain appeared to be aggregated under the experimental conditions chosen by these investigators (92). More recently, Benson & Linderstrøm-Lang have studied whale myoglobin. At pH 7.0 and 37°C., 248 hydrogen atoms of the 264 potentially exchangeable ones exchanged with deuterium. On raising the pH to 8.8, all 264 atoms were replaced (88). From these results it emerges that, in addition to hydrogen atoms that are rapidly and slowly exchangeable, variations of the rate of exchange are to be found within the group of the slowly exchangeable ones (93). This point was investigated in greater detail by Berger & Linderstrøm-Lang (94). With the polyamino acid poly-DL-alanine as a model, they found

that the hydrogen atoms of the NH-peptide bonds exchange at different rates. Urea had no effect on the kinetics, but the reaction was pH-dependent. On the basis of these observations, Berger & Linderstrøm-Lang proposed that the exchange near the ends of the peptide chain occurs by unfolding of the helix, whereas, near the center of the chain, several adjacent hydrogen bonds of the type N—H····O=C, have to be broken simultaneously before an exchange occurs. Poly-DL-alanine has an additional interest for the protein chemist; in this polyamino acid, the D- and L-residues are randomly distributed and the stability of the helix may in part be attributed to internal hydrophobic bonds between the D- and L-methyl groups of the alanine residues rather than to the presence of hydrogen bonds (95, 96). More substances in which the folding is predominantly caused by hydrophobic bonds should, therefore, be investigated. A less complicated picture than that obtained with poly-DL-alanine has been observed by Elliott & Hanby for poly-L-glutamate (97).

The deuterium-hydrogen exchange of subtilisin-modified ribonuclease (RNase-S) (98) and of pepsin-inactivated ribonuclease (99) was recently investigated by Ottesen & Stracher (100). Subtilisin-modified ribonuclease (RNase-S) and its derivative (RNase-S′), obtained by remixing equimolar quantities of RNase-S-Pep and RNase-S-Prot, have the same rate of deuterium exchange, which, however, is faster than that of the intact enzyme. A similar result was obtained if pepsin-inactivated ribonuclease was compared with ribonuclease. The implications of these findings will be discussed in a later section of this review.

Wilcox has studied the deuterium exchange during the reversible denaturation of α-chymotrypsinogen (101). Of the 404 exchangeable hydrogen atoms of bovine α-chymotrypsinogen, a rapid exchange of all hydrogen atoms occurred within 5 to 15 min. if the pH of the solution was lowered to pH 1.5. At pH 3.5, on the other hand, only 360 to 370 hydrogens were replaced by deuterium. From these results, it is apparent that the stability of at least 40 hydrogen bonds in α-chymotrypsinogen is drastically altered when the protein passes from the native to the reversibly denatured state. These findings are of further interest in view of the observations of Schellman (102), who failed to detect any changes of the optical rotatory properties over the pH and temperature range in which the transition from the "native" to the "denatured" state occurs. On the other hand, Stauff & Rasper (103) have discovered small but definite alterations in the light-scattering, specific refractive increment, partial specific volume, and sedimentation constant of this protein during the reversible denaturation. Work of other laboratories has confirmed the results of the Carlsberg group. Different methods have been used and additional data on proteins other than those mentioned above have been obtained (104).

Research on fibrous proteins by Fraser & MacRae, who used the infrared absorption method, indicates that, in silk fibroin, feather rachis, and stretched wool, but not in kangaroo-tail tendon, replacement of the hydro-

gen atoms by deuterium occurs preferentially in the less well-oriented regions of the molecule (105). This was further supported by the studies of Bendit, Feughelman, Fraser & MacRae on stretched keratin (106).

Ultraviolet Spectroscopy

The problem of deciding what role the tyrosine residues of a protein play in maintaining its secondary and tertiary structure has attracted considerable attention from protein chemists during recent years. This interest was stimulated by the classical observations of Crammer & Neuberger (107) who observed that if ovalbumin and insulin are titrated with alkali the absorption maximum is shifted from 275 to 280 mμ to 295 mμ. They ascribed these spectral displacements to the ionization of the tyrosine residues that occurs in alkaline solution. In the case of insulin, all tyrosine residues could be titrated spectrophotometrically at pH 11.0 to 11.5, whereas in ovalbumin the absorption spectrum did not alter greatly over the pH range of 9.5 to 12 but was similar to that recorded at pH 4.9. At pH 13 the spectrum changed very markedly. This was attributed to the presence of anomalous tyrosine residues which only became accessible to titration at higher pH values, i.e., pH 13.0. By use of this method, Fromageot & Schnek (108) found for lysozyme that the tyrosine hydroxyls ionized normally and the spectrophotometric titration was fully reversible. More recently Shugar (109) and Tanford, Hauenstein & Rands (110) demonstrated that three of the six tyrosine residues of crystalline pancreatic ribonuclease are "normal", whereas three have an anomalous behavior. If, however, titration of ribonuclease is carried out in 8 *M* urea and in ethylene glycol, respectively, Blumenfeld & Levy (111) and Sage & Singer (112) found that all six tyrosine residues are ionized and can be titrated in the pH range from 9.5 to 13.0.

From a more extensive study of the spectral behavior of ribonuclease, insulin, ovalbumin, and bovine serum albumin, Tramer & Shugar (113) concluded that one may divide the phenolic hydroxyls qualitatively into at least three groups which may be distinguished by the extent to which they dissociate: (*a*) hydroxyls which ionize normally and have a pK of 10 to 10.4; (*b*) masked hydroxyls which are titrated only after heat or alkali denaturation of the proteins; and (*c*) those which become accessible after rupture of the disulfide bonds. In the case of heat-inactivation of ribonuclease, a stepwise release of "masked" phenolic groups occurs and parallels the loss of enzymic activity. In contrast, on heat denaturation of ovalbumin and serum albumin not all phenolic hydroxyls are unmasked. Although the absorption maximum of ovalbumin solutions at pH 13 is shifted to longer wavelengths (107), Perlmann (114) observed a shift of the maximum from 280 to 275 mμ if the pH of the alkali-denatured ovalbumin was adjusted to pH 6.8. On prolonged exposure to alkali, the absorbancy of the main absorption band, if recorded at neutrality, decreased, indicating that a destruction of the phenolic side chains of the tyrosine residues had taken place. Moreover, the electrophoretic mobility of the major component, A_1, decreased from -5.8 to -5.4×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ (sodium phosphate buffer of pH 6.8 and 0.1 ionic strength). Tramer & Shugar (113) further found that breaking of the

disulfide bonds of ribonuclease, insulin, and bovine serum albumin by performic acid oxidation, in all cases gave products with normally titratable phenolic hydroxyls with apparent pK values of 10.0 to 10.4. Although part of the masking of the phenolic hydroxyls can certainly be ascribed to hydrogen bonding, Tramer & Shugar stress the fact that strong local electrostatic effects are also involved and may account for some of the anomalies observed. Here the reader should be referred to the recent work of Sela & Katchalski (115), who, with the aid of spectrophotometric titrations, studied the behavior of three copolymers, L-tyrosine-L-aspartate, L-tyrosine-DL-alanine, and L-tyrosine-L-lysine, in ratios of one tyrosine to nine of the other amino acid residues. The spectrophotometric titration of the tyrosine-alanine copolymer revealed that the phenolic hydroxyls of this polypeptide resemble in their dissociation those of free tyrosine rather than those of polytyrosine (116). This was explained by the relatively large distance between the tyrosine residues of the copolymer. In contrast, the tyrosine residues in the L-tyrosine-L-lysine ionized more readily than in the L-tyrosine-DL-alanine polypeptide as a result of the strong positive electrostatic field formed by the ϵ-amino groups of the lysine residues. The dissociation of the phenolic groups in the copolymer of tyrosine and aspartic acid, on the other hand, was retarded as compared with the dissociation of L-tyrosine-DL-alanine; this retardation was attributed to the negative electrostatic field formed by the ionized carboxyls. None of the solutions of the above mentioned copolymers showed any spectral change on standing, as had been observed with poly-L-tyrosine, nor did urea have any effect.

In a recent study, Bigelow & Ottesen (117) have compared the spectral behavior of performic acid-oxidized and of pepsin-inactivated ribonuclease. Their investigations extended from pH 1.0 to 11.0. In the case of the former, all six tyrosine residues were titrated at pH 9.8, whereas with the latter two steps that corresponded to a ratio of "free" to "masked" groups of 5:1 were observed in the titration curve. In view of these findings, Bigelow & Ottesen concluded that, during performic acid oxidation, the secondary and tertiary structure of the protein was destroyed, whereas limited pepsin digestion appears to have normalized only two of the three anomalous residues in native ribonuclease.

In contrast to the spectral shifts toward longer wavelengths, which are observed in the pH range of 7 to 12, are those in which the absorption bands of protein solutions are displaced to shorter wavelengths on titration of a protein with acid (118), upon denaturation with urea or guanidine salts (119 to 121), or on mild proteolysis. It has recently become customary to represent these spectral displacements as difference spectra. In the case of a protein containing both tyrosine and tryptophan, three maxima at 277 mμ, 285 mμ, and 294 mμ are present. As shown with the aid of model compounds, the maximum at 295 mμ can be attributed to tryptophan (122), whereas those at 277 and 285 mμ reflect the tyrosine residues (118, 119). Scheraga and co-workers (118, 119, 122), who were the first to use differential spectroscopy in the study of proteins, proposed the hypothesis that these small spectral displacements of 20 to 30 A to shorter wavelengths reflect changes in the

hydrogen bonding of the tyrosyl hydroxyls. In the method of Crammer & Neuberger (107) the protein is treated with alkali to rupture the hydrogen bonds and the ionized hydroxyls of the tyrosine residues are measured spectrophotometrically. In the titration with acid, i.e., pH 6.0 to 1.0, the tyrosine residues remain unionized, but each acceptor group to which the tyrosine hydroxyls is hydrogen-bonded gains a proton; in this case, the acceptor groups are being titrated. The pK's derived from such titration curves indicate the type of groups involved. On the basis of these observations, Scheraga and co-workers suggested that the carboxylates could act as an acceptor group, i.e., $C_6H_5OH\cdots{}^{-}OOC-$.

An alternative explanation, however, follows from the work of Wetlaufer, Edsall & Hollingworth (123) who observed spectral changes of the same order of magnitude as those found with proteins during titration with acid, when the pH of solutions of tyrosine, O-methyl-tyrosine, and glycyl-O-methyltyrosine was changed from 7.0 to 1.0. In these compounds, the occurrence of intramolecular hydrogen bonds can be excluded, and the effects can be ascribed to the ionization of groups adjacent to the phenolic group of the tyrosine residue. Hence, in a protein with tightly folded peptide chains, the extent of ionization of a dissociable group in close proximity to a chromophoric group may influence considerably its spectral properties. The findings of Wetlaufer *et al.* (123), therefore, raise some question as to the validity of the interpretation that the difference spectra observed in the case of proteins are solely caused by hydrogen bonding.

Since the early studies of Laskowski *et al.* (119) on insulin, and of Scheraga (118) on ribonuclease, Leach, Laskowski & Scheraga (124) have compared the spectral shifts obtained when Zn-insulin and Zn-free insulin at pH 8.0 are modified either by acidification or by tryptic digestion. It is of interest to note that the character of the acid-induced shift remained undiminished in magnitude after tryptic digestion. From the change of absorbancy, Leach, Laskowski & Scheraga conclude that two tyrosine residues of the insulin molecule are hydrogen bonded but that only one carboxylate ion acts as an acceptor.

A shift in the pH of pepsin solutions from 4.6 to 1.0 gives rise to spectral displacements (125) similar to those observed in the case of insulin and ribonuclease (118, 119). In view of the high content of tyrosine and of dicarboxylic acids, some of these effects can be ascribed to the occurrence of hydrogen bonding between the aromatic residues and carboxylate ions. In addition, Blumenfeld & Perlmann (125) observed that on mild autolysis of pepsin at pH 2.0 the absorbancy in the ultraviolet further decreased. Since pepsin acts on peptide bonds adjacent to an aromatic residue, new polar groups in close proximity to the chromophore are formed. It appears, therefore, that the decrease in absorbancy observed during autodigestion is most likely caused by the charge effects suggested by Wetlaufer *et al.* (123).

Chervenka (126, 127) has attempted to relate ultraviolet spectral changes to the enzymic activity of chymotrypsin. The spectral changes during the activation of chymotrypsinogen to chymotrypsin involve, predominantly, the absorption of tyrosine residues, and appear to be related to structural

changes in only a limited part of the molecule. This investigator also noticed that a spectral shift accompanied the autolysis of the protein and was of the same order of magnitude as that observed if either chymotrypsin or chymotrypsinogen was treated with urea. During the autodigestion, as well as in the urea treatment of this protein, both the tyrosine and the tryptophan residues are involved in the displacement of the absorption maximum.

A similar study was carried out by Smillie (128) who studied the changes in the ultraviolet absorption spectrum of trypsin; here again, spectral displacements were observed during the autolysis of the protein in absence of Ca^{++} or when the protein was treated with urea. Since the difference spectrum of trypsinogen in 7.5 *M* urea was found to be similar to that of trypsin, Smillie concluded that the spectral changes did not involve the active center of the enzyme. Had that been the case, changes should have occurred both during activation of the zymogen and during the reaction of trypsin with diisopropylfluorophosphate.

Williams & Foster (129), in their study of bovine plasma albumin by differential spectroscopy, favor the view that the perturbation of the spectrum results from changes in the molecular structure of the protein that are known to occur in the pH range wherein the carboxyls are titrated. With bovine serum albumin the changes in the absorbancy do not parallel protonation of the carboxylic groups. An increase of the ionic strength or replacement of the chloride ions of the solvent by thiocyanate results in a decrease of the absorbancy rather than in the increase one would expect if the hydrogen-bonded phenolic hydroxyls were bound to carboxylate ions.

From the foregoing, it is apparent that the small shifts observed in the ultraviolet spectra of proteins can arise from a variety of causes, e.g., an alteration in the hydrogen-bonding of a protein, electrostatic effects, or the formation of ionizable groups in the vicinity of a chromophoric group. To arrive, therefore, at unequivocal conclusions as to the underlying mechanism in each case necessitates that the experimental evidence obtained from the ultraviolet measurements be supplemented by the results of other chemical and physicochemical studies as has been done in the study of denaturing agents on ribonuclease (120, 121).

TERTIARY STRUCTURE

Effects of Disulfide Bonds

The bonds that play a major role in maintaining the tertiary structure of proteins are undoubtedly the —S—S— linkages between two half-cystines in the primary structure. Since the pairing of these residues determines the number of configurational possibilities of the peptide chain, the disulfide bonds are important in "locking" parts of the protein molecule into the conformation essential for its biological activity. Modification of proteins caused by rupturing —S—S— bonds, either by oxidation or reduction, is, therefore, one of the key problems in protein chemistry. Since this question has been reviewed by Linderstrøm-Lang & Schellman (5), reference here will only be made to a recent article by Kauzmann (131). With the aid of statistical principles used in polymer chemistry, Kauzmann calculates the number of

isomers that can arise if a protein contains more than one cystine residue. When applied to native ribonuclease and native insulin, it could be concluded that isomers in these proteins are neither among the most probable nor among the least probable, whereas native serum albumin may be one of the more probable isomers. Such analysis can, of course, only be carried out if not too many disulfide bonds exist in the protein.

X-Ray Diffraction Studies

One of the landmarks in elucidation of the tertiary structure of proteins is undoubtedly the elegant work of Kendrew and his collaborators (132, 133) on myoglobin. Myoglobin consists of 153 amino acid residues, linked together in a single chain, and a prosthetic group. Using the method of isomorphous replacement, first introduced by Perutz and colleagues in their study on hemoglobin (134), Kendrew *et al.* compared the x-ray patterns of crystalline myoglobin before and after introduction of the heavy atom Hg at different sites in the molecule. From the observed intensities of the reflections, they computed an electron density map of the contents of the unit cell of the crystals and thus were able to obtain a full three-dimensional representation of the molecule. Based on a resolution of 6 A, they were able to trace the polypeptide chain and to locate the heme group in the electron density map. They also constructed a model ($42 \times 35 \times 25$ A) in which the polypeptide chain is arranged in a highly irregular and complicated manner. The length and shape of the polypeptide chain implies that, in most parts, it is folded, and it was estimated that 70 per cent of the chain is present in a helical conformation. This conclusion is supported by the results of Benson & Linderstrøm-Lang on the deuterium exchange of myoglobin (88), discussed elsewhere in this article. As illustrated with the aid of Figure 2, the most striking feature of the structure, however, is its irregularity. As pointed out by Kendrew and his colleagues, the model represents the contours of the chain, and, in order to obtain more information on the location of the side chains, electron density maps of higher resolution, e.g., 1 to 2 A, will have to be obtained. If such is possible, the x-ray method will undoubtedly become the most powerful method in the elucidation of the tertiary structure of even more complicated proteins. As already shown by Perutz (135), progress is being made with hemoglobin, and some of his results foreshadowed the recent chemical investigations of Itano and Singer.

Another globular protein which is being studied with the aid of the x-ray diffraction technique is ribonuclease (136). Recently, a low-resolution investigation of chymotrypsinogen has been initiated by Kraut (137).

QUATERNARY STRUCTURE

Before concluding the general section of this review, a fourth level of organization, the quaternary structure should be introduced. Although its existence has tacitly been recognized for many years, it has only recently been named by Bernal (138). This concept will be of particular importance for the more complex types of molecules formed by association of molecular units that are already folded. Hemoglobin is a typical example (135). More

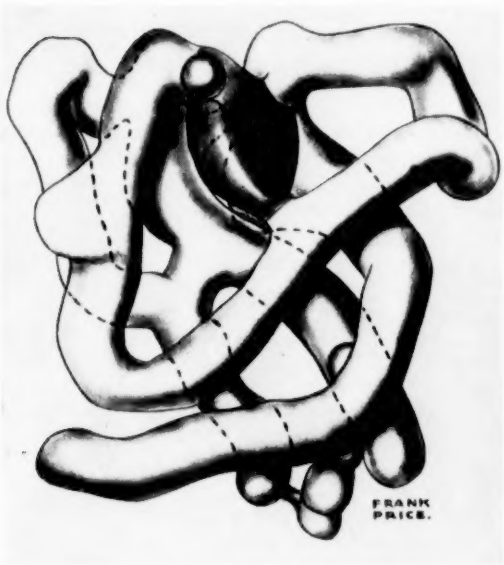

FIG. 2. Drawings of a model of the tertiary structure of the myoglobin molecule, derived from the three-dimensional electron density map. (Courtesy of Dr. J. C. Kendrew.)

recently, Green & Aschaffenburg (139) have shown that β-lactoglobulin consists of two subunits, and Harrison (140) has suggested that ferritin, a protein with a molecular weight of 747,000, should be composed of 24 (or 24 n) subunits.

VARIOUS PROTEINS

RIBONUCLEASE

The primary structure of ribonuclease as known up to 1958 was described in the review by Hill, Kimmel & Smith (3). Since that time, Hirs, Moore & Stein (4) and Hirs (141) have completed, by use of the type of methods described in last year's review (3), a determination of the amino acid sequence in the performic acid-oxidized ribonuclease molecule. Hirs obtained from the entire length of the peptide chain milligram quantities of purified peptides which served for the later sequence determinations. The following changes were subsequently made (4): the aspartic acid at position "15" was changed to asparagine, the asparagine-serine sequence at position "23–24" was reversed, the glutamine at position "49" was changed to glutamic acid, and the serine-glycine sequence at position "89–90" as well as the glutamine-alanine sequence at position "102–103" were reversed. They suggested that, although there were no conflicting results in

their study, the proposed formula, nevertheless, should be taken as a working hypothesis—with a multitude of determinations required, a chance for error was created. Moreover, the importance of using highly purified proteolytic enzymes for sequence studies was stressed.

There are four disulfide bridges in ribonuclease. Performic acid oxidation, which had been successfully applied to elucidation of the structure of insulin, was also used for the localization of disulfide bridges in the peptides obtained from ribonuclease. Ryle & Anfinsen (142) established linkages between half-cystine residues I and VI, II and VIII, III and VII, and IV and V. In a preliminary report, Spackman, Moore & Stein (143) reported that they could only isolate peptides containing cysteic acid residues I and VI and IV and V, respectively, after the intact ribonuclease molecule had been degraded with trypsin and chymotrypsin.

In a more recent investigation, Spackman, Stein & Moore (144) studied the stability of the disulfide linkages and found that they were most stable in the pH range 2 to 6.5. Also, N-ethylmaleimide, generally used to minimize disulfide interchange, seemed to increase the rate of disappearance of parent disulfides. They demonstrated that prolonged incubation at pH 7 in the presence of N-ethylmaleimide and 2 *M* guanidine hydrochloride, as employed in their work for the hydrolysis of ribonuclease by trypsin and chymotropsin, labilized the disulfide bonds. The lability also varied with the given disulfide. Hence, they chose to degrade ribonuclease with pepsin at pH 2, followed by trypsin and chymotrypsin at pH 6.5. With the aid of these conditions, they isolated, in 20 to 50 per cent yields, peptides containing the half-cystine residues I and VI, II and VII, III and VIII, and IV and V. They suggested that the low yields resulted from the fact that enzymatic cleavages did not proceed quantitatively to give a single cystine-containing peptide involving each disulfide bond. In Figure 3 the complete amino acid sequence of ribonuclease is presented.

The method of splitting disulfide bonds is of extreme importance if the modified protein is to be used in sequence studies. Moore *et al.* (145) discussed the reduction of several proteins, including ribonuclease, by sodium borohydride and the reaction of the reduced proteins with iodoacetic acid. Methionine recoveries were of the order of 90 per cent. Bailey & Cole (146) investigated the conversion of disulfide bonds to two S-sulfonate groups in the presence of iodosobenzoate or tetrathionate as oxidizing agents. This reaction had the advantages of mildness and extreme specificity, but different proteins varied considerably in their reaction rate. Bailey & Cole (146) pointed out that a kinetic investigation should be carried out before the method is adopted for any particular protein. Sela, White & Anfinsen (147) achieved quantitative reduction of ribonuclease (and also of lysozyme) at room temperature with thioglycolic acid at pH 8.5. Finally, Gundlach, Stein & Moore (148) studied the reaction of methionine with iodoacetate. They came to the conclusion that in the alkylation of reduced proteins, as short a reaction time as possible should be used, e.g., about 20 min., in order that formation of the carboxymethylsulfonium derivative of methionine

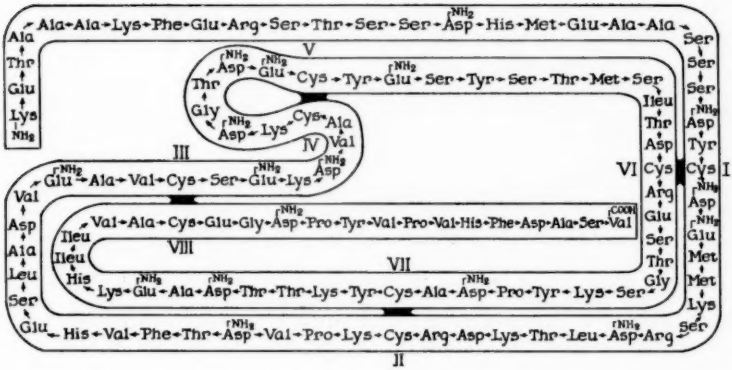

FIG. 3. A diagrammatic representation of the sequence of amino acid residues of bovine pancreatic ribonuclease, based on the information presented by Hirs, Moore & Stein (4). (Diagram, courtesy of Dr. S. Moore.)

would be insignificant. Unfortunately, the reaction with methionine is pH-independent and proceeds in the range 2 to 8.5.

One of the ultimate aims of the biochemist is to relate structure to function. Since much is known about the primary and secondary structure of ribonuclease, investigators have been pursuing such a correlation of the different properties of this protein. Several approaches have been tried. Anfinsen and his group (149, 150) have compared the composition and partial amino acid sequence of bovine and ovine pancreatic ribonuclease. They reasoned that differences in the amino acid sequence should reflect noninvolvement in enzymic activity since the action of these two enzymes *in vitro* is the same. They found that position "3" in the beef enzyme was occupied by serine in the sheep enzyme, and the lysine at position "37" in the beef enzyme by glutamic acid in the sheep enzyme. Another as yet undefined difference was found around positions "99 to 104." Since the replacement of lysine by glutamic acid would cause an abrupt change of charge, this particular portion of the molecule would, therefore, not be involved in the active site of the enzyme.

Another approach that has been followed is the study of activity remaining after chemical modification of the enzyme molecule. Richards & Vithayathil (151) have shown that the action of subtilisin on ribonuclease at pH 7.0 to 8.0 ruptures the peptide bond between positions 20 and 21 and results in the formation of a peptide (S-peptide) and new protein (S-protein). When the two products were remixed in equimolar proportions, almost the full original activity was regained. Although the association of S-peptide and S-protein was very strong at neutral pH, the complex was different from the original molecule, as demonstrated by the increased susceptibility of the complex to trypsin inactivation. This is highly suggestive that the secondary

structure is altered, as is also reflected by changes in the optical rotatory properties (152) and by an alteration in deuterium exchange as compared to the native enzyme (100).

Resnick, Carter & Kalnitsky (153) reported that, by use of a controlled method for the cleavage of disulfide bonds (154), 20 per cent of the activity of ribonuclease was lost if two —S—S— bonds were ruptured, whereas a 40 per cent loss occurred if three disulfide bonds were hydrolyzed. On cleavage of 3.5 bonds, the enzyme became inactive. However, they did not mention the urea concentrations at which these determinations were carried out, a striking omission in view of the fact that, in the presence of 6 *M* urea, appreciable activity remained when 3.9 disulfide bridges were cleaved, while no activity remained if this was done in the presence of 8 *M* urea.

White & Anfinsen (155) used an ingenious isotope method for following the reduction of specific disulfide bonds. It was established that the 1–6 disulfide bond was the most labile and is probably not essential for ribonuclease activity.

Gundlach, Stein & Moore (156) studied the nature of the amino acids involved in the inactivation of ribonuclease by iodoacetate. When the inactivation was carried out at pH 5.5, one of the products was a chromatographically pure protein that was totally inactive and differed from ribonuclease A only in that the imidazole group of a single histidine residue had been carboxymethylated. The involvement of a histidine residue in the active center of ribonuclease was also reported in a preliminary note by Barnard & Stein (157). Ribonuclease was inactivated by alkylation with bromoacetic acid. Cytidylic acid, a competitive inhibitor, gave effective protection against the inactivation, indicating an active center reaction with the alkylating agent.

Brown *et al.* (158) studied the antigenic structure of ribonuclease. They found that ribonuclease A and B reacted identically and that the disulfide bonds were essential for antigenic activity.

From the foregoing, it is apparent that, although the primary structure of pancreatic ribonuclease has been elucidated and much information on the secondary and tertiary structure of this enzyme is available, no indication has yet been obtained as to what determines its specificity and enzymic activity per se.

Lysozyme

Another protein which has attracted the attention of many protein chemists is lysozyme. Since last year's report (3), sequence studies on this protein have progressed considerably. Jollès, Jollès-Thaureaux & Jauregui (159) analyzed 17 peptides separated from a chymotryptic digest of heat-denatured hen's egg lysozyme. Most of these peptides had an aromatic amino acid as the C-terminal residue. This study has increased to 105, the number of amino acids that could be assigned positions within the peptides. Simultaneously, Ando, Fugioka & Kawanishi (160) have determined the C-terminal sequence of this protein and found, both with carboxypeptidase and hydrazinolysis, that leucine is the C-terminal residue. Since, in the case

of oxidized lysozyme, arginine and alanine were also liberated, Ando and co-workers assigned the sequence Ala·Asp (NH_2)·Arg·Leu to the C-terminal end of the polypeptide chain. Jollès & Ledieu (161) determined the molecular weight and the amino acid composition of dog spleen lysozyme. Lysine was shown to be the N-terminal amino acid and leucine the C-terminal residue, as is the case in hen's egg lysozyme.

That lysozyme is a compactly folded molecule (162) follows from its low intrinsic viscosity. At urea concentrations higher than 5.0 to 6.0 *M*, lysozyme undergoes swelling, but, as judged from the changes of the specific optical rotation, $[\alpha]_D$, unfolding occurs only at a urea concentration higher than 7.0 *M*. It is of considerable interest that the alterations in the hydrodynamic properties of this protein precede those in the optical properties. The studies on the secondary structure have recently been extended by Glazer who investigated the effect of pH on the denaturation of lysozyme (163). From viscosity and optical rotation measurements, it was concluded that lysozyme shows greatest stability towards urea and guanidine hydrochloride at pH 4 to 8. Addition of cysteine to the protein in 8 *M* urea at pH 9.5 greatly increased the viscosity, thus indicating disulfide bond involvement in the native structure of the enzyme.

In an investigation carried out simultaneously, Beychok & Warner (164) studied the rate of denaturation of lysozyme as a function of pH. They concluded that the extreme stability of the enzyme in acid solution was not attributable to hydrogen bonding and suggested that it may be caused by the presence of the disulfide bridges.

That the polypeptide chain of lysozyme does not have a highly helical conformation follows also from the work of Elliott, Hanby & Malcolm (165). Elliott *et al.* were able to measure the optical rotatory dispersion of lysozyme films cast from aqueous solution and to compare it to the infrared spectrum in the region of the carbonyl-stretching mode on specimens prepared under identical conditions. The optical rotatory properties give no dispersion characteristics indicative of α-helices. It does not follow, however, from the work on films, that the polypeptide chains must have the same conformation when present in crystals.

Papain

Considerable progress toward the elucidation of the amino acid sequence of papain has been made by Smith and collaborators (166) who used methods similar to those described for the structural studies on ribonuclease and lysozyme. Since this work has been adequately described by Hill, Kimmel & Smith (3) and is also discussed elsewhere in this volume (167), only certain pertinent facts concerning the primary structure of papain will be mentioned here. On fractionation of oxidized papain, Kimmel *et al.* were able to characterize peptides with overlapping sequences, which account for 110 of the 180 amino acid residues. With this information on hand, they succeeded in allocating partial sequences of 31 residues from the N-terminal end, 25 from the C-terminal end, and 33 from the center portion of the polypeptide chain (168).

On the basis of the presently available information on the activity and the "active site" of papain (169, 170), Smith has proposed a mechanism of action for this enzyme, which is well corroborated by our present knowledge of the chemical and kinetic behavior of this protein. In view of the fact that a free thiol group does not appear to be present in active papain, Smith suggested that a "high energy" bond exists between a thiol ester of an unionized sulfhydryl group and a carboxylate ion. This thiol ester is formed and maintained through the energy derived from the folded conformation of the peptide chain. It is clear, of course, that a model such as that proposed by Smith implies that papain is a compactly folded molecule. The observations of Hill, Schwartz & Smith (171), however, indicate that in addition to hydrogen bonds other factors determine the tight structure of this protein.

In a recent study on the effect of urea and guanidine hydrochloride on the activity of papain, Hill, Schwartz & Smith (171) observed that, at low concentrations of these reagents, e.g., below 1.0 *M*, the enzyme was reversibly inactivated. No inactivation was observed at intermediate concentrations of urea and guanidine salts. In 6.0 *M* urea the enzyme loses activity, although little change of the specific optical rotation, $[\alpha]_D$, is observed. Here, the inactivation is reversible. In 5.0 *M* guanidine hydrochloride, on the other hand, the loss of enzymic activity is irreversible and is accompanied by an increase of the levorotation. Papain, therefore, differs from other proteolytic enzymes in that the inactivation at low concentrations of the so-called hydrogen bond-breaking reagents is not accompanied by a drastic change in the optical rotatory properties of the protein. However, the enzyme resembles other proteins in that it is affected by high concentrations of guanidinium ions, both with respect to its catalytic function and its specific optical rotation. The extent to which an unfolding of the peptide chain occurs, or delineation of the type of bonding necessary in the folded structure for the biological activity of papain, cannot be inferred from these measurements alone without additional information obtained by other methods.

Pepsin

The chemistry and biological activity of pepsin and the activation of its zymogen have recently been reviewed by Neurath (172, 173). Thus far, amino acid sequence work on this protein has not been attempted, which may be partly attributed to the size of the molecule, i.e., pepsin has a molecular weight of 35,000. Moreover, the homogeneity of crystalline pepsin has not yet been established unambiguously. Although homogeneous in the ultracentrifuge, in the electric field, and on chromatography on columns of diethylaminoethylcellulose, crystalline pepsin may represent a family of closely related enzymes. Questions of this kind are particularly pertinent when a new function of an enzyme is being discovered. Neumann and coworkers observed recently a pepsin-catalyzed transpeptidation in a mixture of, for example, N-acetyltyrosyltyrosine and pepsin. This transpeptidation involves an amino transfer to a free carboxylic group as an acceptor (174, 175). No clear decision can be made at present whether pepsin has both hydrolytic and transpeptidation activity or whether they reside in chemi-

cally distinct proteins. It is of interest, however, to point out that the discovery of Neumann *et al.* may, in part, explain the failure of previous investigators to obtain a 100 per cent hydrolysis if dipeptides were used as synthetic substrates in pepsin assays (176 to 178).

Recent work of Blumenfeld & Perlmann on the amino acid composition of crystalline pepsin has indicated some impurity with respect to the hydroxyproline (179). It is quite possible that this amino acid originates from a contaminating peptide, such as a breakdown product of collagen. A close examination of the amino acid distribution of pepsin reveals the presence of 44 aspartic acid, 27 glutamic acid, and 15 proline residues, a high content of hydroxyamino acids and non-polar residues and only 4 basic ones, i.e., one lysine, one histidine, and two arginines. Three disulfide bonds serve as cross links of the single peptide chain. This new amino acid analysis differs from that previously reported by Brand (180).

Although studies on the primary structure of pepsin are almost nonexistent, a few observations pertaining to the secondary structure have been reported recently. With the aid of a potentiometric titration of the native and denatured pepsin, Edelhoch (181) has demonstrated the appearance of new titratable groups if pepsin is denatured in solutions more alkaline than pH 6.0. The liberation of protons parallels the rate of inactivation of the enzyme, and the pH zone of acid released is decreased if the ionic strength of the medium is increased. The types of weak bonds which conform best with these results are hydrogen bonds between a carboxyl group acting as a donor and a second one as acceptor, —COOH·····⁻OOC—. Edelhoch (182) studied also the effect of guanidine hydrochloride, urea, metallic cations, ethanol, and detergents on pepsin inactivation in the neutral range. Inactivation in the presence of urea and guanidinium ion was attributed to the weakening of the carboxyl (donor) hydrogen bonds, in contrast to the hydrophobic bonds affected by ethanol.

In an attempt to define the conformation of the peptide chain segments necessary for the biological activity of the enzyme, Blumenfeld, Léonis & Perlmann (183) have investigated the effect of guanidine hydrochloride and urea on the activity of pepsin in acid solutions. The zone of maximum stability in aqueous solution (pH 2 to 5) is narrowed to pH 3.0 to 3.5 in concentrated guanidine hydrochloride and to pH 3.3 to 4.3 in urea. At pH values below 3.0, loss of activity in the presence of hydrogen bond-breaking reagents occurs rapidly and the formation of non-protein nitrogen caused by autodigestion parallels the rate of inactivation. In solutions more alkaline than pH 5.0, the loss of activity in the presence of urea and guanidine salts is rapid and little non-protein material is formed. Since the increase of the intrinsic viscosity is small and no significant change of the optical rotatory properties has been observed, Blumenfeld *et al.* conclude that a marked unfolding of the pepsin molecule does not occur.

A synthesis of the presently available facts leads to the conclusion that, although hydrogen bonds of the type C=O·····H—N and others that involve the phenolic hydroxyls of tyrosine and the carboxylate ions of acidic amino acid residues, $C_6H_5 \cdot OH$·····⁻OOC—, undoubtedly exist in pepsin (43,125),

they must be relatively unimportant for its enzymic activity. It is further concluded that the helical content of pepsin is negligible and that the peptide chain is in a "random coil" conformation (43). It would be of interest to test whether the newly discovered parapepsins of Ryle (184, 185) and the gastricin of Tang *et al.* (186) exhibit a similarly anomalous behavior toward hydrogen bond-breaking reagents.

The recent discovery of proteins with peptic activity (184 to 186) reopens the controversy of the homogeneity of pepsin. Ryle (184) and Ryle & Porter (185), by use of chromatography on diethylaminoethylcellulose, isolated two new enzymes from crude preparations of pig gastric mucosa and named them parapepsin I and parapepsin II. Although similar to pepsin in some of their physicochemical properties, in their low pH optima, and in their sites of attack on the B-chain of oxidized insulin, these enzymes differ from pepsin in their N-terminal residues, their stability at pH 6.9, and their activity against various synthetic substrates. A summary of the properties of pepsin and parapepsins I and II is given in Table V.

Whether a relation of the parapepsins to gastricin exists is not clear at present. Tang *et al.* (186) isolated from gastric juice the enzyme "gastricin" and crystallized it. It proved to be homogeneous on ultracentrifugation and on zone electrophoresis on starch gel and differs from pepsin in three properties: (*a*) pH optimum of pH 3.0; (*b*) electrophoretic migration on paper; and (*c*) heat inactivation. Gastricin, moreover, has a lower milk-clotting activity than pepsin. A further chemical characterization of this protein will be needed to define unequivocally the relationship of the various "pepsin-like" enzymes.

In view of the recent paper by Funatsu & Tokuyasu (187), it is of interest that Tang *et al.* (186) suggested that gastricin may possibly be a product of decomposition of pepsin. Funatsu & Tokuyasu reported the isolation of an "active fragment" obtained by autolysis of crude pepsin at pH 4.0 and 3° to 5°C. for 48 hr. This fragment was subsequently separated by dialysis and had a sedimentation constant (S) of 0.71, and a molecular weight of less than 10,000 (cf. 188). Here the reader should be referred to the discussion on "active fragments" presented by Hill, Kimmel & Smith (3) and by Linderstrøm-Lang & Schellman (5).

Hemoglobin

Titrimetric methods have given various values for the number of sulfhydryl groups per molecule of hemoglobin. Cole, Stein & Moore (189) determined the total content of half-cystine by several methods. The protein was oxidized with performic acid or alkylated with iodoacetic acid, and the resulting cysteic acid or S-carboxymethyl-cysteine was determined chromatographically. Also, the spectroscopic change in N-ethylmaleimide-treated globin was measured. Amperometric titrations with silver ions were carried out on the same samples of hemoglobin and globin as used for the other determinations. The values seemed to cluster around five residues of half-cystine per molecule, which could be interpreted as four to six residues per molecule. Higher values were obtained by the amperometric technique, but these were

TABLE V

SUMMARY OF PROPERTIES OF PEPSIN AND PARAPEPSINS I AND II*

	Parapepsin I	Parapepsin II	Pepsin
$S_{20,w}$	3.26	3.32	3.0 to 3.16
Molecular Weight	38,600	40,700	34,500
N-terminal Amino Acid Residue	Ala	Ser+Leu	Ileu
Phosphorus (atoms/mole)	0.03	0.0	1.0
Stability at pH 6.9	Stable	Unstable	Very unstable
pH Optima	1.8	1.8; 3.0	1.5 to 2.0
Inhibition by Pepsin Inhibitor	−	+	+

* See (185).

interpreted as an excess binding of silver at sites other than the sulfhydryl groups of cysteine residues.

Since the last review on this subject (3), several papers have been published on the chemical differences between normal hemoglobin and several abnormal hemoglobins. This work is particularly exciting because it describes a biochemical manifestation of genetically differentiated defects. One of the major advances was the availability of a method for the separation, on a preparative scale, of the two types of hemoglobin subunits. Wilson & Smith (190) developed a method for the fractionation of valyl-leucyl- and valyl-glutamyl-polypeptide chains of horse globin by their adsorption from formic acid solution to Amberlite CG50 type 2, followed by elution with urea solutions at pH 2. One peak was obtained for valyl-leucyl-globin and two peaks for valyl-glutamyl-globin. This procedure was adapted for human hemoglobin by Hunt (191) and Ingram (192). Hill & Craig (193) recently reported the successful separation of the α- and β-chains of human globin by countercurrent distribution.

Hunt separated the chains of isolated adult (A) and foetal (F) human hemoglobins. The fractions were subjected to tryptic digestion followed by chymotryptic hydrolysis of the trypsin-resistant cores, and the resulting peptides were separated by the "fingerprint" technique of Ingram. The "fingerprint" of the fraction first eluted from the Amberlite column was found to be identical for hemoglobins A and F. These A_1 and F_1 fractions were also found to be identical by other criteria, namely, specific amino acid tests, tyrosine-tryptophan ratios, and N-terminal DPN determinations. From the latter, Hunt was also able to conclude that the fraction first eluted must correspond to the α-chain of hemoglobin A. "Fingerprints" of the last eluted fractions demonstrated, on the other hand, many differences between hemoglobins F and A.

Ingram (192) and Maruyama & Ingram (194) showed that hemoglobins S, C, D_β, and E differ from hemoglobin A in the β-chain, while D_α and I differ from hemoglobin A in the α-chain. These differences were previously reported by the above workers and were also reviewed by Hill, Kimmel &

Smith (3). Vinograd and co-workers (195) substantiated that hemoglobins A and S differ in the β-chain.

A comparison of the α- and β-chains of various hemoglobin types has also been carried out by a dissociation and recombination technique. Singer & Itano (196) demonstrated such a recombination by associating unlabeled and labeled molecules. Hemoglobin molecules were labeled in two ways. In the heme-labeling method, where carbonmonoxyhemoglobin and ferrihemoglobin are mixed, the results could be analyzed by electrophoresis on the basis of total charge differences. In the second method, C^{14}-labeled hemoglobin was prepared by the isolation of hemoglobin from individuals who had been injected with C^{14}-labeled glycine. They were able to conclude that there was an exchange of fragments of hemoglobin molecules in acid solution and that each type of hemoglobin molecule dissociated into two non-identical subunits (each containing two identical polypeptide chains).

Itano & Robinson (197), using this ingenious method, showed that hemoglobins A, I, and S dissociated at pH 4.7 and reassociated at pH 6.3 to give one new component, more hemoglobin A, and less hemoglobin I and S. A similar experiment with hemoglobins A, I, and C gave no new electrophoretic component, but A increased, and I and C decreased. For the sake of discussion, they gave to the β-chains of hemoglobins A, S, and C, the designations a, s, and c, respectively. They called their common α-chain u, and the abnormal α-chain of I, i. Then asymmetric dissociation of a_2u_2, $s_2u_2^{+2}$, $c_2u_2^{+4}$, and $a_2i_2^{-4}$ would give a_2, s_2^{+2}, c_2^{+4}, u_2, and i_2^{-4}.

Jones, Schroeder & Vinograd (198) reported that the α-chains of hemoglobins F and S could interchange, as could the α-chains of hemoglobins A and F after dissociation in acid or alkali. But, in neither case was hybridization complete. This suggested to them that the forces between the chains of the various hemoglobins were different.

It had been shown that hemoglobin H has four N-terminal valyl residues per molecule, and only one N-terminal sequence, namely, Val·His·Leu. This sequence defines β-chains and suggests that hemoglobin H could be represented as β_4^H. Vinograd and co-workers (195, 199) proceeded to show, by the hybridization technique, that β^H-chains and β^A-chains (β-chains derived from hemoglobin A) are identical. They treated carbonmonoxyhemoglobin H and radioactive carbonmonoxyhemoglobin S to induce hybridization. Their results are summarized below (*indicates a labeled protein):

Hemoglobin type	H	+	S*	→	H	+	S*	+	A*	+	β_4^{S*}
Formula	β_4^A		$\alpha_2^{A*}\beta_2^{S*}$	→	β_4^A		$\alpha_2^{A*}\beta_2^{S*}$		$\alpha_2^{A*}\beta_2^A$		β_4^{S*}

Furthermore, chromatographic studies, sedimentation velocity determinations, and examination of the N-terminal peptides substantiated that only the α-chains of the resulting hemoglobin A were radioactive.

Collagen

As pointed out in an earlier section of this review, a relatively large amount of proline and hydroxyproline is present in collagen, particularly in the amino acid sequence —Gly·Pro·Hypro—. On the basis of x-ray diff-

fraction, two similar models, collagen I and collagen II, have recently been proposed for collagen (200 to 203); these models are essentially modifications of a structure published by Ramachandran & Kartha (204). In this structure, three polypeptide chains, each coiled in a left-handed helix of the type described by Cowan & McGavin for poly-L-proline II (205) and by Crick & Rich for polyglycine II (206), are held together by intrachain hydrogen bonds to form a three-stranded helix. Although the x-ray diffraction and the infrared absorption properties of collagen would not alone permit a differentiation of model I and model II, the thermal stability of different collagens (207 to 209) would be compatible with the existence of two different structures. On gentle heating of collagen in acid solution (209 to 211), or by use of a high salt concentration (209 to 212), a dissociation into the three polypeptide chains occurs. Although the conformation of the individual chains approaches that of a random coil, rotatory dispersion measurements reveal that some helical conformation is still present (45). On the other hand, if a gelatin solution (gelatin being the transformation product of collagen) is cooled below the critical temperature, a configuration resembling that of native collagen is regained.

In an attempt to supplement the physicochemical studies on the secondary structure of collagen and gelatin, Harrington, Von Hippel & Mihalyi (213) have initiated an investigation on the molecular conformation of ichthyocol gelatin by use of the proteolytic enzyme collagenase as a "structural probe". The purification, the chemical and physicochemical properties, as well as the assay of collagenase from *Clostridium histolyticum* (an enzyme with a molecular weight of 109,000, a sedimentation constant of 5.4 S, and a diffusion constant of 4.3×10^{-7} cm.^{2} sec.$^{-1}$), have been described extensively by Seifter, Gallop and co-workers (214, 215). Thus far, it has been found that collagenase acts only on collagen and gelatin. The enzyme, however, hydrolyzes copolymers of glycine and proline with the formation of N-terminal glycine, but it is inactive toward Ala·Gly·Gly—, Leu·Gly·Gly—, Gly·Gly·Gly—, and poly-L-proline (216). On the basis of these results, Seifter and co-workers suggested that collagenase has a specificity towards the amino acid sequence —X·Gly·Pro·Y—. The observations of Kazakova, Orekhovich & Shpikiter (217) are in accord with these findings. However, the recent work of Nagai & Noda (218) indicates that the specificity of the enzyme may be broader than was assumed originally.

If, as shown by Von Hippel & Harrington (219), the kinetics of proteolysis of ichthyocol gelatin solution is followed by combining measurements of the optical rotation and of the number of peptide bonds broken, the proteolysis follows first-order kinetics. As stated above, under these conditions, the protein is a single chain molecule in a random coil conformation. On lowering the temperature from 30° to 5°C., the kinetics of the proteolysis is more complex and is best explained if one assumes that two independent reactions occur simultaneously. Although both follow first-order kinetics, their rates differ markedly. On the basis of these results, also supported by viscosity and light-scattering measurements, Von Hippel & Harrington proposed the following model for the gelatin collagen-fold transition: (*a*) The primary step

which occurs on cooling of the gelatin solution is a local intrachain conformational change and consists of "locking" the proline-containing regions of the peptide chain into the poly-L-proline II conformation. This is reflected in the instantaneous change of the kinetics of proteolysis. (*b*) After establishment of the "locked" proline-containing regions, the remaining parts of the polypeptide chain fold into the loose poly-L-proline II type helix, as reflected by an increase in the levorotation (46). (*c*) After formation of the helix, specific association between the individual helices occurs, and the collagen-type structure is established. It is of interest that, in 1955, Cohen suggested that formation of the helix precedes the association of the peptide chains (44).

Although the model of Von Hippel & Harrington is still of a tentative nature, these investigators have demonstrated that, whereas the action of proteolytic enzymes on protein substrates is largely determined by their specificity toward certain peptide bonds, the rate and the extent of hydrolysis depend on the secondary structure of the protein. For example, the location of the susceptible bonds—whether in a highly folded or unfolded portion of the peptide chain—is of some consequence (5). Moreover, these investigators have shown how an understanding of the behavior of a polyamino acid, such as poly-L-proline, is of great importance in the study of the secondary structure of proteins.

In a survey such as this, the subject matter of which covers widely different fields, the selection of the material to be reported cannot but express, in part, the personal views of the reviewers. This does not excuse, as we know, our failure to discuss much interesting work, and we apologize to the authors whose papers were omitted.

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CHEMISTRY OF AMINO ACIDS AND PEPTIDES[1,2]

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The previous review (1) has covered the literature until November, 1958; consequently, this review deals with approximately the first half of 1959. The number of articles published during this period about equals the total for 1958, an indication of the exponentially increasing activity in the field.

Some excellent lectures and reviews have been published during the last few months; they may help to compensate for deficiencies in this present treatment of the literature: "Chemistry of Polypeptides. Peptide Syntheses IV" [Wieland (2)]; "Amino Acid Sequences in Proteins" [Tuppy (3)]; "Naturally Occurring Peptides and Their Biologic Significance" [Synge (4)]; "Chemistry of Insulin" [Sanger (5)]; "The Relation of Chemical Structure to the Biologic Activity of Pituitary Hormones" [Li (6)]; "The Structure of Antigens and the Nature of Immunological Specificity" [Westphal (7)]; "Peptidic Structure and Its Significance for Catalytic and Biochemical Reactions" [Lautsch (8)]; and "Approaches to Biosynthesis of Proteins" [Meister (9)].

ANALYTICAL METHODS

Sequential Analysis of Peptides

Progress in sequential analysis of peptides is marked by the application of mass spectrometry to the elucidation of amino acid sequence in small peptides and by work that attempts the selective cleavage of peptides and proteins by chemical means. The latter methods, in certain respects, simulate the action of chymotrypsin without, however, sharing the stereochemical specificity of the enzyme.

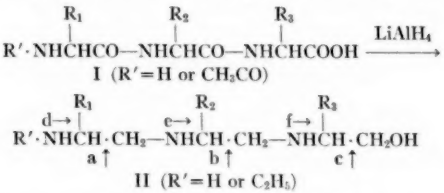
Mass spectrometry.—Biemann, Gapp & Seibl (10) have described a first approach to sequential analysis by mass spectrometry. Should it be possible to refine and extend the method to more complicated peptides—nine di- and three tri-peptides of glycine, alanine, leucine, proline, phenylalanine, serine, and aspartic acid, in quantities of fractions of a milligram, have served as models—this method may be expected to prove most valuable for rapid determination of the structure of small peptides obtained in degradative procedures.

In the method presently employed, the peptide in question [(I) R′ = H]

[1] The survey of the literature pertaining to this review was concluded in July, 1959.

[2] The following abbreviation is used in this chapter: ACTH for adrenocorticotropic hormone.

or its N-acetyl derivative [(I) $R' = CH_3CO$] is reduced to the corresponding polyamino alcohol (II) by means of $LiAlH_4$. The product is subjected to mass spectrometry; the incidental rupture of labile bonds (a, b, c, d, e, f) produces charged particles, the molecular weights of which may be very precisely determined by the positions of the ensuing peaks in the spectrogram.



A peak corresponding to the molecular weight (plus one unit) of the amino alcohol II (product arising from an ion-molecule collision) is usually observed. From this, the molecular weight of the parent peptide may be calculated. Fragments arising from different cleavages appear at the positions indicated in Table I.

TABLE I

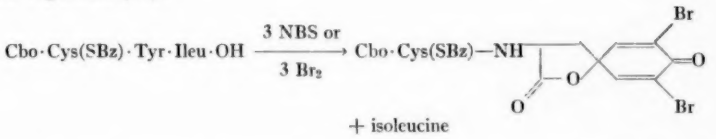
MASS SPECTROMETRY OF TRIAMINO ALCOHOLS II

Point of cleavage	Peak positions of fragments (*molecular weight units*)
No cleavage (ion-molecule collision)	$(R'+R_1+R_2+R_3+143+1)$
a	$(R'+R_1+28)$ (R_2+R_3+115)
b	$(R'+R_1+R_2+70)$ (R_3+73)
c	$(R'+R_1+R_2+R_3+112)$, (31)
d	$(R'+R_2+R_3+143)$, (R_1)
et cetera	

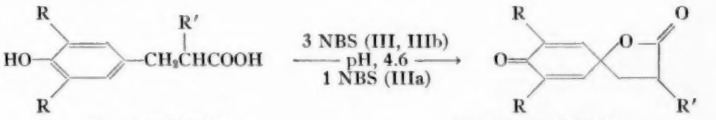
Careful analysis of the spectrogram provides the necessary information for determining the structure. This may, of course, be facilitated and substantiated by qualitative amino acid analysis of the hydrolysate.

Selective chemical degradation.—Subsequent to work on the oxidative cleavage of tryptophyl-amide bonds (1), Schmir, Cohen & Witkop (11) and, independently, Corey & Haefele (12) have demonstrated that tyrosine undergoes a similar reaction with N-bromosuccinimide (NBS) or with bromine in

aqueous media. This oxidation proceeds more rapidly than that of produced, or already existing, amino groups. The reaction explains the hitherto enigmatic cleavage of oxytocin by bromine as observed by Mueller, Pierce, & du Vigneaud (13):



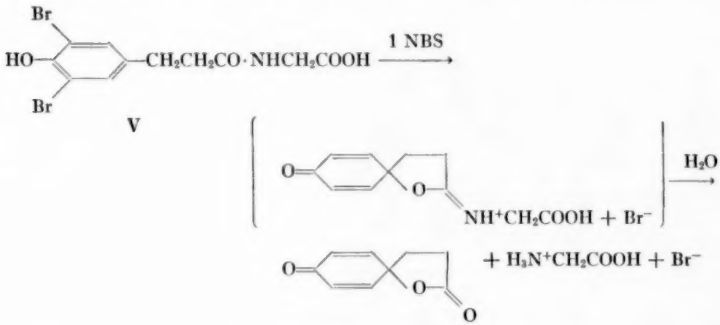
The mechanism of the reaction was studied by using simple derivatives of tyrosine and of the analogous phloretic acid (III). Phloretic acid (III) or dibromo phloretic acid (IIIa) react with bromine or N-bromosuccinimide to give a dibromo spiro dienone lactone (IV). The conversion may be followed by observing the marked change of the ultraviolet spectrum. N-benzoyl tyrosine (IIIb) reacts similarily, yielding the corresponding α-benzoylamino derivative (IVa):



III (R=R′=H)
IIIa(R=Br, R′=H)
IIIb(R=H, R′=—NHCOC$_6$H$_4$)

IV (R=Br, R′=H)
IVa(R=Br, R′=—NHCOC$_6$H$_4$)

N-(dibromophloretyl)-glycine (V) gives a maximum yield of glycine (~80 per cent) with exactly one equivalent of N-bromosuccinimide:



All reactions proceed readily at low temperatures, for example, at −10° in aqueous methanol (12); therefore, besides cleaving tryptophyl and tyrosyl bonds in peptides, they are also useful for the selective removal of phloretyl- or dibromophloretyl-blocking groups in peptide synthesis (11).

Methods for the Isolation and Qualitative and Quantitative Determination of Amino Acids and Peptides

Most of the papers that have appeared during the period under review deal with refinements or applications of existing techniques, and a wealth of methodological and bibliographic information may be obtained from them.

Two developments are of practical interest. Porath & Flodin (14) have advocated the use of a hydrophilic, but insoluble, cross-linked dextran in granular form as a "molecular sieve" for separations according to molecular weight in a column process called "gel filtration." The very promising product that has been used for desalting, exchange of buffer, and group separation of proteins, peptides, and amino acids is commercially available under the name of "Sephadex" (Pharmacia, Uppsala, Sweden). Hannig (15) has developed a simplified, semi-automatic apparatus for the quantitative determination of amino acids; the apparatus is based on the well-known principle of the machine of Spackman, Moore & Stein and is commercially available (Bender and Hobein, Munich, Germany).

GENERAL SYNTHETIC PROCEDURES

Amino Acids

Progress in this field is characterized by: (*a*) the use of chelates for the total synthesis of amino acids and especially for the asymmetric synthesis of threonine; (*b*) the use of N-carboxy alanine anhydride for the synthesis of S-benzyl-D,L-cysteine; and (*c*) the partial synthesis of optically active basic amino acids as well as of derivatives of L-glutamic acid (preparation of L-glutamine and of L-glutamic acid γ-ethyl ester).

Syntheses via chelates.—Overberger & Lusi (16) have demonstrated that the reversible addition of carbon dioxide to nitro alkanes may be forced to completion by simultaneous chelation with magnesium ion. Magnesium methyl carbonate, formed by the action of carbon dioxide on magnesium methylate in dimethyl formamide solution, adds normal nitro alkanes (C_1 to C_4) to give a magnesium chelate of the homologous α-nitro carboxylic acid. This may be decomposed to the free α-nitro carboxylic acid and catalytically hydrogenated to the corresponding α-amino acid.

Akabori and his colleagues (17) have extended the Knoevenagel-type condensation of acetaldehyde with the copper chelate of glycine to glycine-cobalt complexes. The α-chelate gives better yields than the β-form, and the ratio of the product (D,L-threonine/D,L-allothreonine) is greater than with the copper method. Condensation at 70° for 2 hr. gives a total yield of 32 per cent with a ratio[3] of threonine to allothreonine of 7.6. Effective catalysts are Na_2CO_3 and K_2CO_3; Na_2HPO_4 and sodium acetate are ineffective.

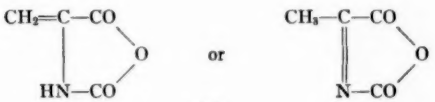
Murakami & Takahashi (18) have reacted the laevo form of glycino-bis-(ethylenediamine)-cobalt iodide with acetaldehyde in the presence of sodium carbonate to give an 80 per cent yield of crude crystalline amino

[3] This ratio is temperature-dependent.

acids with an overall dextro rotation. Paper chromatography revealed threonine, allothreonine, and glycine in a ratio of 7:2:1; the asymmetric yield was 8 per cent.

Should it be possible to develop the reactions so as to produce the expensive optically active forms of the β-hydroxyamino acids in substantial quantities, this line of research, besides having theoretical value, may also prove to be of practical interest.

N-carboxy dehydroalanine anhydride.—Sakakibara (19) has shown that pyruvic acid reacts with two molecules of benzyl urethane to give the dicarbobenzoxy derivative of β-diamino propionic acid, which can be decomposed to N-carbobenzoxy dehydroalanine. From this, N-carboxy dehydroalanine anhydride (VI)



VI

may be produced in the usual manner by the action of PCl_5. One of the most interesting properties of this very reactive compound is its capacity to add on benzyl hydrogen sulphide, eventually producing S-benzyl-D,L-cysteine.

Basic amino acids.—According to Zaoral & Rudinger (20), optically active α,γ-diamino butyric acid and ornithine (and from this, arginine) are easily available, starting from N-tosyl- or carbobenzoxy- L-asparagine and L-glutamine methyl esters. Intermediates are the corresponding side-chain nitriles, obtained by dehydration, which are catalytically hydrogenated to the corresponding amines. Cleavage of the tosyl group is effected by means of HBr in acetic acid at 70° to 80°. Zaoral (21) has developed a total synthesis of D,L-α,γ-diamino butyric acid from γ-butyro lactone—a method which is in some respects similar to the approach of Talbot, Gaudry & Berlinguet (22).

Derivatives of glutamic acid.—Rudinger and his colleagues (23) have improved the preparation of L-glutamine, starting from N-tosyl-L-glutamic acid and proceeding via tosyl-L-pyroglutamic acid, as described earlier by himself (24) and by Swan & du Vigneaud (25). A mode of preparation of γ-ethyl L-glutamate that is claimed to be reliable and reproducible is described by Pravda (26).

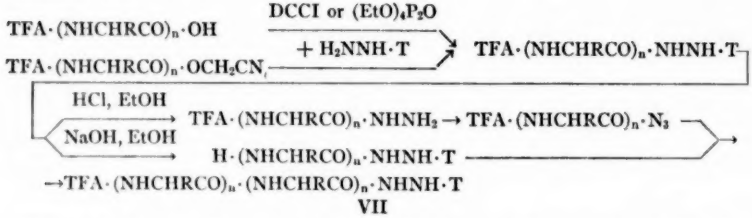
Peptides

Much work is still being done on the general synthesis of peptides from amino acid derivatives. All methods follow more or less the classical approach of introducing protecting groups that have to be selectively removed during the course, or at the end, of the synthesis. The ideal method of condensing free amino acids in the correct sequential order and in aqueous media has not yet been found.

Protection of functional groups.—The work of Weygand has yielded still

more information about amino group protection with the increasingly important trifluoroacetyl group. Weygand & Rinno (27) have described N-trifluoroacetyl serine and N-trifluoroacetyl threonine. From these, they have prepared a number of seryl- and threonyl- dipeptides and tripeptides. The trifluoroacetyl group, which is usually removed by dilute alkali, in these cases may very rapidly be cleaved by the action of HCl in ethanol.

The facile removal of the trifluoroacetyl group by dilute alkali has led Weygand & Steglich (28) to the use of this group, in combination with trityl hydrazine, in an ingenious manner, the general idea of which is shown in scheme[4] VII.



VII

The synthetic procedure may be terminated by use of an amino acid or peptide ester as the amino component in the last stage, followed by removal of the amino and carboxyl protective groups by alkaline hydrolysis. The method has great advantages, because it offers the possibility of using the trifluoroacetyl group in conjunction with the azide method, which is known to be safe in respect to partial racemization.

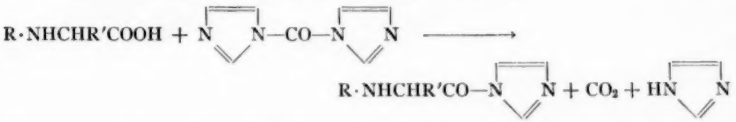
The introduction of the trityl group into amino acids has been the subject of a study by Stelakatos, Theodoropoulos & Zervas (29). The group is becoming increasingly popular in peptide synthesis as it can easily be removed by mild acid treatment. Hitherto, the preparation of trityl amino acids has been rather tedious; it involved the introduction of this group into amino acid esters, followed by saponification under drastic conditions because of the strong steric hindrance introduced by the trityl residue. The present method condenses trityl chloride with amino acids in aqueous-organic solutions containing diethylamine; the diethylamine salts of the trityl amino acids are then extracted into chloroform. The yields, considering the amount of unreacted amino acid recovered, are claimed to be good. Among others, ditrityl histidine, N^{α}-trityl histidine, N^{im}-trityl histidine methyl ester, and O-trityl tyrosine benzyl ester have been described.

The protection of the guanidino function of arginine still presents a challenge to the peptide chemist, although nitro (30) and tosyl (31) groups have been used. Zervas *et al.* (32) have now published *in extenso* their work on tricarbobenzoxy arginine and arginyl-arginine [mentioned in the preceding review (1)].

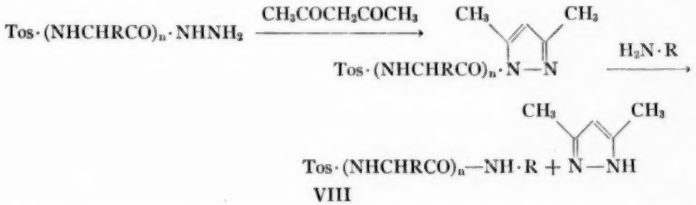
[4] The following abbreviations are used: DCCI for dicyclohexyl carbodiimide; T for trityl-; TFA for trifluoroacetyl-; n for 1, 2, 3. . . .

Methods of condensation.—Three papers have appeared that again revert to the use of acid chlorides as reactive carboxyl components. Rieche & Gross (33) have treated a mixture of phthaloyl amino acid and amino acid ethyl ester hydrochloride with asymmetric dichloro dimethylether. On heating, HCl is evolved and the protected dipeptide is produced. The method has not been applied to even moderately complicated peptides, and the problem of partial racemization has not been studied. This applies also to a method of cyclization that uses peptide chloride hydrochlorides as intermediates [Rothe, Brünig & Eppert (34)].

Following the appearance of papers by Staab (35) and Anderson & Paul (36), the use of carbonyl diimidazole as a condensing agent in peptide synthesis is becoming increasingly popular. Very probably, a substituted amino acyl imidazole is produced as a reactive intermediate:



Giesemann & Braun (37) have shown that this reagent reacts with free amino acids to give, in the case of phenylalanine, N,N′-carbonyl-diphenylalanine. Ried & Schleimer (38) have prepared analogous reactive amino acyl heterocycles by another reaction (VIII).

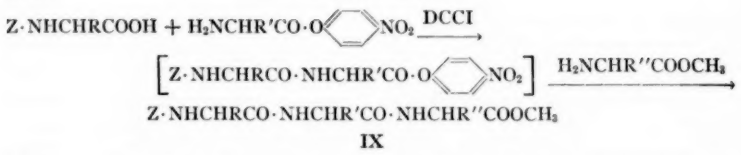


VIII

These derivatives are less reactive than the corresponding amino acyl imidazoles [cf. also Staab (39)], and conversion to the amide requires heating. A number of simple di- and tripeptides were prepared by this method. The same authors (40) have studied the reaction with various amines and have arrived at conclusions concerning the influence of nucleophilicity of the amine and of catalysis by weak acids on the rate of reaction similar to those previously reached by Schwyzer and co-workers (41) in earlier work on active esters.

Active esters have been used in an ingenious manner by Goodman & Steuben (42) for an abbreviated synthesis of tripeptide derivatives. The idea is shown in reaction sequence[5] IX.

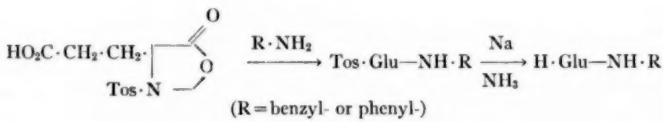
[5] The following abbreviations are used: DCCI for dicyclohexyl carbodiimide; Z for carbobenzoxy-.



IX

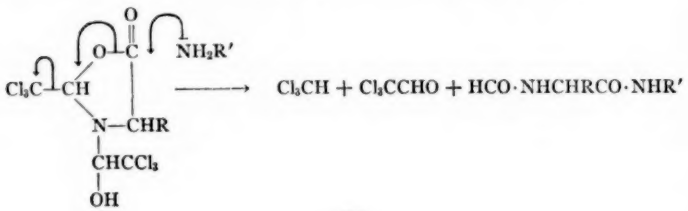
Any reagent for condensation other than dicyclohexyl carbodiimide, provided its reaction velocity is much greater than that of side reactions resulting from the amino acid active ester (e.g., the mixed anhydride procedure), may be used. The intermediate dipeptide is not isolated but immediately reacts with another amino acid ester. The yields are extremely good, purification of the product is easy and, in the examples studied, no racemization has been observed.

Micheel & Haneke (43) have extended earlier work on the use of oxazolidone-type intermediates to the synthesis (X) of α-amides of glutamic acid.



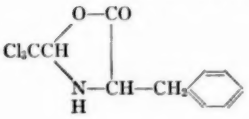
X

Unfortunately, the production of oxazolidone intermediates is, in this procedure, confined to N-tosyl amino acids. This drawback of introducing a protecting group that is not easily removed is avoided in a similar procedure by Dane, Heiss & Schäfer (44). These authors have prepared oxazolidone derivatives from amino acids and anhydrous chloral. During the subsequent reaction with an amino component, a retro-aldol condensation reaction takes place and N^{α}-formyl peptides (see XI) are produced (the reaction was studied with H·Gly·OEt and H·Gly-Gly·OEt as amino component):

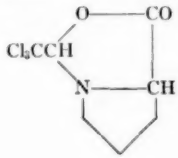


XI

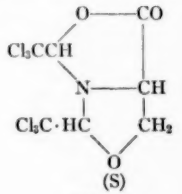
The reaction products of chloral with phenylalanine (XII), proline (XIII), serine (XIV), and cysteine (XIV) have a somewhat different constitution but react very similarly with amines.



XII



XIII



XIV

Panneman, Marx & Arens (45) have made a very careful study of the use of ethoxy ethyne in peptide synthesis. Dipeptides are obtained in an optically pure state and in good yields by boiling equivalent amounts of N-protected amino acids and amino acid ester hydrochlorides with ethoxy ethyne in ethyl acetate containing 0.5 per cent of water. For the preparation of higher peptides, it is preferable to add amino components, not as the hydrochlorides, but as free esters, because otherwise partial racemization may occur. Carbobenzoxy glycyl phenylalanine is already partially racemized by the action of ethoxy ethyne in the boiling solvent, indicating that the presence of a nitrogenous base is not necessary for this side reaction to occur.

Cyclization.—Morozova & Zhenodarova (46) have prepared the cyclic hexapeptide cyclo·Gly·Leu·Gly·Gly·Leu·Gly, which already has been described by Kenner, Thomson & Turner (47) and by Schwyzer & Gorup (48). The latter authors have observed that not only Gly·Gly·Gly azide (49), but also a number of other reactive derivatives (i.e., active esters) of tripeptides and even pentapeptides [Schwyzer & Sieber (50)], tend to dimerize under conditions of cyclization. This behavior has been ascribed to the relief of steric strain encountered in cyclic tripeptides (47, 50, 51) and to the possibility of special preferred conformations in transition states and in products derived from the interaction of two peptide intermediates that comprise an odd number of amino acid residues (50, 51).

Varia.—The preparation of a number of peptides by various known methods has been reported. Schnabel (52) has prepared the heptapeptide Ser·Gly·Ala·Gly·Ala·Gly·Tyr (L_4). Brand's rule was applied to the molecular rotation of this and other peptides. X-ray powder diagrams, as well as infrared spectra, indicate the β-conformation in the solid state. Schellenberg & Ulbrich have prepared a number of α- and γ-peptides of glutamic acid with glycine and tyrosine as other components (53).

RESOLUTION OF RACEMATES, DETECTION OF DIASTEREOISOMERS, AND PARTIAL RACEMIZATION

These three topics are of great importance for preparative amino acid and peptide chemistry. Losse and his co-workers (54, 55) have made further studies on their method of resolution by use of D-tartaric acid and derivatives,

especially dibenzoyl-D-tartaric acid. They have carried out the resolution of a number of amino acid amides and hydrazides as well as of a few dipeptide esters containing one asymmetric center. Wegmann (56) has used both dibenzoyl-D-tartrate and dibenzoyl-L-tartrate for the resolution of *p*-bromo phenylalanine, thus obtaining equally insoluble stereoisomeric salts of both the D- and L- forms of the amino acid ester. Vogler & Lanz (57) have described a powerful method for the resolution of D,L-threonine by means of the diastereoisomeric brucine salts of D- and L- phthaloyl threonine (total yields of 60 to 70 per cent). The enzymic resolution of β-methyl aspartic acid by means of acylase II has been described by Benoiton *et al.* (58).

Unfortunately, no progress has been recorded on the detection of small amounts of stereoisomers of amino acids (in synthetic peptides), a problem which is becoming of great importance for polypeptide synthesis.

Schnabel (59) has confirmed earlier observations on the extraordinary ease with which residues of serine and threonine in peptides are racemized in alkaline media. He has converted carbobenzoxy D-seryl-glycyl-L-alanine methyl ester to carbobenzoxy D,L,-seryl-glycyl-L-alanine methyl ester by the action of triethylamine in methanol. At this stage, the diastereoisomers may be separated by crystallization, thus allowing the conversion of all of the D-L isomer to the L-L form.

GENERAL REACTIONS OF AMINO ACIDS AND PEPTIDES

A fundamental prerequisite for our understanding of the biochemical reactions of amino acids and peptides is a thorough knowledge of their chemical reactivity. It is to be hoped that, with increasing availability of more complicated synthetic peptides and polypeptides, a growing number of chemists will turn their attention to the complex problems presented. Current studies involve: (*a*) various chemical reactions of the functional groups and stereochemical influences of side chains upon these reactions; (*b*) catalysis of model substrate reactions by amino acids and peptides—a field which should be supplemented by fundamental studies on polyfunctional catalysis —; (*c*) the study of complexes and chelates of hydrogen and metal ions; and (*d*) the study of the influence of radiation (including spectral properties)—a field closely connected with the study of radiation injury.

Various chemical reactions.—Oxidation of sulfur-containing compounds still presents an important problem. Lanthionine-S-oxide and -S-dioxide in aqueous solution undergo β-elimination reactions which may introduce complications into structural studies [Zahn & Baschang (60)]. The oxidation product of cystine is not, as was believed, cystine disulphoxide but has the structure of the corresponding thiolsulphonate [Grushko & Guryanova (61); Sweetman (62)]. This structure explains in a very rational manner the infrared spectrum and reactions of the compound, e.g., that with cysteine, leading to cystine plus alanine-β-sulfinic acid.

Zervas *et al.* (63) have studied disulphide interchange reactions on simple unsymmetrical open-chain derivatives of cystine and have shown that the

interchange is base-catalyzed. An extremely useful "by-product" of this study is the preparation of L-cystine in an optical purity of over 99.9 per cent by the action of renal D-amino acid oxidase.

Ludwig & Hunter (64) and Petersen & Tietze (65) have demonstrated that amino groups of amino acids and peptides may react with imido-esters to give the corresponding amidines. The reaction has preparative value (synthesis of carboline alkaloids) and might prove helpful in analytical studies.

A study that may lead to prediction of properties of the enzymic system which converts diiodotyrosine to thyroxine has been undertaken by Matsuura & Cahnmann (66), who have shown that β-(3,5-diiodo-4-hydroxyphenyl)-propionic acid, an analogue of diiodotyrosine, is specially suitable for this purpose: the compound gives an 11 per cent yield of the thyroxine analogue in aqueous solution at pH 7.7 to 7.9 on standing for a period of seven days. Mechanistic studies of this important reaction are under way.

Catalysis of model substrate reactions.—The mechanism of the presumably bifunctional catalysis of triglyceride hydrolysis by lysine has been the subject of controversy [Chesbro & Hedrick vs. Gero (67)], but the final word does not seem to have been spoken. Catalysis of the hydrolysis of *p*-nitro phenylacetate by imidazole, histidine, and related compounds has been the subject of much study. Koltun, Clark & Dexter (68) have shown, for example, that the reaction is equally well catalyzed by imidazole and carbobenzoxy prolyl-histidyl-glycine amide. Zn(II) and Cu(II) complexes are inactive; their stability constants may be determined by measuring the amount of catalysis exerted. *p*-Nitro phenylacetate acetylates the nitrogen of the diglycine anion in aqueous solution; measurement of the rate of reaction provides a convenient means of determining diglycine-anion concentration in systems containing Zn(II) and Cu(II) [Koltun & Gurd (69)].

Complexes with hydrogen and metal ions.—Complex formations, which often have been a source of trouble for the preparative peptide chemist, have been studied by Grassmann & Wünsch (70), Goodman & Steuben (71), and Grommers & Arens (72). These authors have shown that carbobenzoxy-amino acids and -peptides may, under certain conditions, form complexes with their alkali and ammonium salts (often crystalline). These reactions may become valuable for our understanding of the binding of such cations by proteins.

Other valuable studies of the stability and reactivity of metal (and hydrogen) complexes of amino acids and peptides have been reported [Fakagi, Chihara & Seki (73); Datta, Leberman & Rabin (74); Leberman & Rabin (75); Datta & Grzybowsky (76); Trapmann (77); Bamann, Rother & Trapmann (78); Biester & Ruoff (79)].

Interaction with radiation.—Bennett & Garrison (80) have observed the production of amide groups and of ammonia in the radiolysis of proteins. Peter (81) has studied the reaction products of the irradiation of lysine. Baliah & Rangarajan (82) have postulated a possible intramolecular proton

transfer during the electronic excitation of acetanilide to explain the spectral properties. This might have some bearing on our notions of the fine structure of the peptide bond in the *cis* configuration. Ellenbogen (83) has studied the infrared spectra of peptides and amino acids in aqueous solution and has found that many bands present in the solid state disappear, whereas the fundamental frequencies of =CO, =NH, and =CH remain. With increasing concentration, the bands of water (3 and 6μ) disappear, possibly because of the "freezing" of H_2O molecules around the dipoles of the substance.

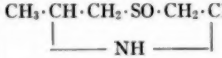
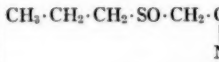
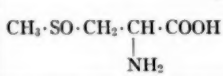
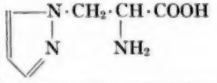
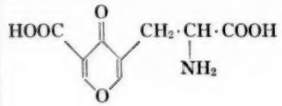
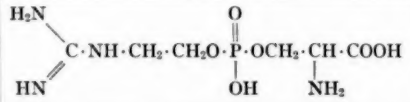
NATURALLY OCCURRING AMINO ACIDS AND PEPTIDES

Amino Acids

Some of the newly isolated amino acids of natural origin are listed in Table II. The application of nuclear magnetic resonance to the elucidation of structure of compound No. 5 is a unique feature. γ-Amino-β-hydroxy butyric acid (No. 1) was shown to be present in brain and to inhibit transmission of

TABLE II

Naturally Occurring Amino Acids

No.	Structure	Name, source, other indications	Reference
1	$H_2NCH_2 \cdot CHOH \cdot CH_2COOH$	γ-Amino-β-hydroxy butyric acid; brain (synthesis reported)	(84)
2		Cyclo-alliin; *Allium* species (synthesis reported)	(85)
3		Dihydro-alliin; *Allium* species	(86)
4		S-Methyl-cysteine sulphoxide; *Allium*	(86)
5		Pyrazolyl alanine; *Citrullus vulgaris*	(87)
6		Stizolobic acid; *Stizolobium hassjoo*	(88)
7		Lombricine; *Lumbricus* (earthworm)	(89)

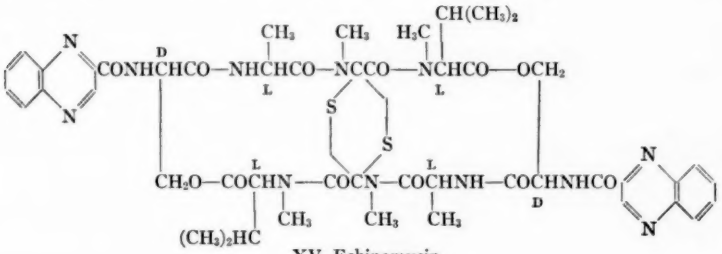
the nerve impulse in a manner similar to that of Florey's fraction I. It also antagonizes the convulsions induced by injection of sodium glutamate and -citrate into the motor area of the cerebral cortex.

In addition to the compounds listed, Heyns & Paulsen (90) have isolated a number of fructose- and glucose amino acids from aqueous extracts of mammalian liver. N-[D-fructosyl-(1)]-glycine, -L-alanine, and -L-valine were obtained in crystalline state; the corresponding derivatives of L-serine, L-threonine, L-aspartic acid, L-leucine, L-tyrosine, L-methionine, and L-proline were either isolated in the amorphous form or otherwise identified. In addition N-[D-glucosyl-(2)]-glycine was isolated. Similar derivatives of peptides also seem to be present in liver extracts.

The absolute configuration of (-)-α-methyl-β-alanine (from human urine) has been deduced by Balenović & Bregant (91), by relay to (-)-2-methylbutanol, and found to be that of (R)-α-methyl-β-alanine [$COOH \cdot HC(CH_3) \cdot CH_2NH_2$]; for the definition of R cf. Cahn *et al.* (92).

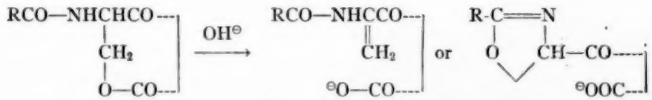
Peptides

Antibiotics.—One of the highlights of the chemistry of antibiotic peptides during the period under review is the work of Keller-Schierlein, Mihailovic & Prelog (93) on the structure of echinomycin (XV).



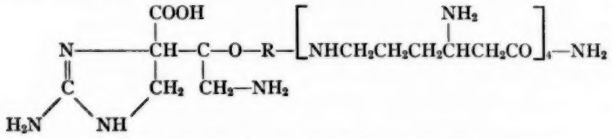
XV. Echinomycin

The proposed bicyclic dilactone-ring system and the presence of a heterocyclic aromatic acid residue relate echinomycin to the actinomycins and etamycin and may have implications on the formulae of the actinomycins [Brockmann & Boldt (94)]. A very interesting feature of echinomycin is the possible dithiane ring. Alkaline hydrolysis of echinomycin to echinomycic acid (XVI) is not a simple process of saponification; moreover either a β-elimination (at the serine residues) or the formation of an oxazoline ring from serine seems to be involved.



Echinomycin XVI. Echinomycic Acid

Another antibiotic substance from actinomycetes, geomycin IV, may be formulated as in XVII [Brockmann & Cölln (95)].

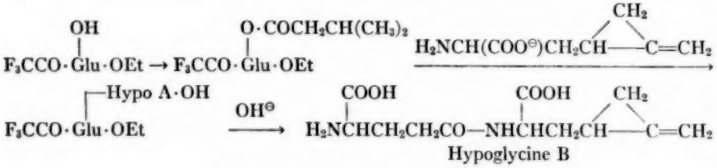


XVII. Possible Structure[6] of Geomycin IV

Further studies on the amide and carboxyl groups of bacitracin A have been reported [Swallow & Abraham (96)]. Subtilin A has been isolated by partition chromatography on silica gel [Alderton & Snell (97)], and characterization studies, undertaken by Stracher & Craig (98), have shown that the molecular weight (by partial substitution) is 3300; the amino acid composition[7] is Asp, Pro, Gly_2, Ala, Val, Ileu, Leu_4, Phe, Lys_3, Lan, β-Me-Lan_4, Glu_3, Try, Sar_2. Subtilin seems to be a pentacyclic peptide with a side chain containing sarcosine as N-terminal amino acid.

Work on the total synthesis of penicillin V has been published *in extenso* [Sheehan & Henery-Logan (99)]. Schwyzer & Sieber (100) have reported on the synthesis of a protected pentapeptide sequence contained in tyrocidin A that involves the use of colored protecting groups and the 4-nitrobenzyl residue.

Toxic peptides.—The constitution of hypoglycine B, a second toxic and pharmacologically active component from *Blighia sapida*, has been recognized as γ-glutamyl-hypoglycine A (1, 101) by Jöhl & Stoll (102) and Hassall & John (103). Confirmatory syntheses have been carried out in both laboratories. The synthesis by the Swiss group [which has been reported in full detail (104)] may be outlined as indicated in reaction sequence XVIII.



XVIII. Synthesis of Hypoglycine B

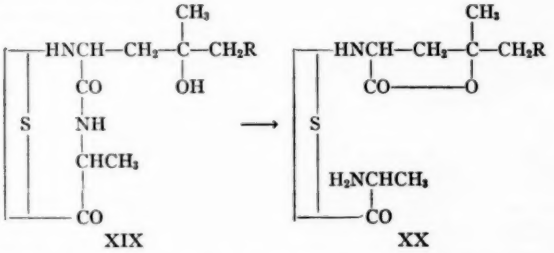
Hassall & John have used the condensation of N-trityl glutamic acid and hypoglycine A by means of dicyclohexyl carbodiimide to establish the γ-peptide bond (steric hindrance of the α-carboxyl group by the trityl residue).

More work on the toxic peptides of the mushroom *Amanita phalloides* has

[6] R in this formula is a carbohydrate residue, $C_{18}H_{37-39}N_2O_{12}$.

[7] Lan (lanthionine); Sar (sarcosine).

been reported by Wieland and co-workers. Acid hydrolysis of α-amanitin yielded a new amino acid, β-methyl-γ,δ-dihydroxy leucine [Wieland & Höfer (105)]. According to the most recent investigations, phalloidin contains D-threonine and, instead of the formerly postulated δ-hydroxy leucenine, the hydrated form of this amino acid, L-erythro-γ,δ-dihydroxy leucine. This uncommon amino acid is replaced by γ-hydroxy leucine in phalloin and by α-amino-γ-oxo valeric acid in ketophalloin [Wieland & Schöpf (106)]. The ring system of phalloin and phalloidin may be opened selectively by hot dilute sulfuric acid to form a γ-lactone, as shown in formulae[8] XIX and XX; this cleavage occurs at the peptide bond emanating from the carboxyl of one of the hydroxylated leucines.



Wieland, Freter & Gross (107) have reported on very interesting synthetic experiments in this field. Their plan was first to build the heterodetic bridge by condensing S-chloro derivatives (sulfenic acid chlorides) of peptides containing cysteine with tryptophan peptides and then to close first one, and afterwards the other, loop of the bicyclic ring system by establishing the corresponding peptide bonds. The first and second steps have been carried out on model compounds with success.

Hormones and "tissue hormones."—Acher, Chauvet & Lenci (108) have shown that horse and lamb oxytocins exhibit no structural peculiarities that would differentiate them from the oxytocins of other investigated species and that the vasopressins belong to the arginine series [as was also demonstrated by Light & du Vigneaud (109) in the case of the human hormone]. Bodanszky & du Vigneaud have accomplished two new syntheses of oxytocin. The first (110), of which full details are available, resembles the synthetic approaches to the vasopressins. The second (111) stresses the long known and already successfully applied fact [e.g., Schwyzer & Li (31); Boissonnas *et al.* (112); Schwarz & Bumpus (113)] that partial racemization during peptide synthesis may be avoided by starting the synthesis at the carboxyl end and by adding one carbobenzoxy amino acid after the other, proceeding in a stepwise manner towards the amino end; the synthesis is remarkable in that

[8] R in phalloidin is OH and in phalloin is H.

active esters of the carbobenzoxy amino acids are used as the reactive species throughout.

Work on the biological action of vasopressin has indicated that the attachment at the renal receptor site may possibly be effected by disulphide bonds [Fong *et al.* (114)]. Direct arterial perfusion of adrenal glands of the dog has shown that synthetic lysine-vasopressin stimulates the secretion of hydrocortisone [Hilton *et al.* (115)].

One of the most remarkable accomplishments this year was the synthesis of the acetyl-tridekapeptide-amide, α-MSH (α melanophore-stimulating hormone from porcine pituitaries), by Guttmann & Boissonnas (116), which now has been reported in full detail. Removal of the carbobenzoxy- and benzyl-protecting groups in the last step was effected by means of HBr in trifluoroacetic acid, thus avoiding acetylation of hydroxyl groups of serine and tyrosine.[9] A protected octadecapeptide with the amino acid sequence of beef β-MSH has been synthesized by Schwyzer and co-authors (117). The compound,[5] Z·Asp(NH_2)·Ser·Gly·Pro·Tyr·Lys(Tos)·Met·Glu(NH_2)·His·Phe·Arg·Try·Gly·Ser·Pro·Pro·Lys(Tos)·Asp($OMe)_2$, elicits somewhat more than 1 per cent of the activity of the natural hormone.

Steelman, Anderson & McGregor (118) describe a simplified procedure for the isolation of the α and β melanophore-stimulating hormone from the by-products of ACTH manufacturing. Varon has found a substance that resembles melanophore-stimulating hormone to be present in human placenta, thus making the already very complicated picture of the specificity of peptide "hormones" even more enigmatic (119). The work of Harris (120) and of Harris & Roos (121, 122) on the structural elucidation of these α and β hormones from pig pituitary glands has appeared in detail and presents a wealth of information on analytical peptide chemistry.

Li (123) has proposed a very versatile system of terminology, using subscripts and superscripts, for preparations of adrenocorticotrophic hormones. The system takes into account the possibility of finding natural or synthetic analogues of known preparations of ACTH. Léonis, Li & Chung (124) have studied the action of a preparation of chymotrypsin on α-corticotropin and have found that, besides bond cleavage at the expected points, other peptide bonds are also attacked. The acid-base equilibria of α- and bovine corticotropin and a discussion in terms of amino acid sequence have been presented by Léonis & Li (125). Panneman, Marx & Arens (45) have synthesized, by use of ethoxy ethyne as agent for condensation, the hexapeptide H·Pro·Val·Lys·Val·Tyr·Pro·OH which comprises the amino acid sequence in position 19 to 24 of ACTH.

Schwarz & Bumpus (113) have described in detail the synthesis of the

[9] The synthesis of a strongly active derivative of α melanophore-stimulating hormone that contains the residues of glutamine and of N^{ϵ}-tosyl lysine instead of those of glutamic acid and of lysine has been announced [Hofmann, K., Yajima, H., and Schwartz, E. T., *Biochim. et Biophys. Acta*, **36**, 252–53 (1959)].

optically pure tetrapeptide derivative[5] Z·Arg(NO_2)·Val·Tyr·Ileu·OMe, used in their previous synthesis of Ileu(5)-angiotensin (horse), by a variety of methods. Walaszek & Huggins (126) have reported on the occurrence of a hypertensive agent (substance A) which they claim to be a polypeptide in fraction IV-4 of plasma protein. Here again is an example of different peptide "hormones" exhibiting similar pharmacologic effects when the pressor activity of the hypertensins (angiotensins) and of peptides like "pepsitensin" is considered.

A number of procedures for the isolation and purification of polypeptide hormones has been published: interstitial cell stimulating hormone from sheep pituitary glands [Squire & Li (127)], ovine lactogenic hormone [Cole & Li (128)], mammogenic hormone of the anterior pituitary gland [Damm (129)], parathyroid hormone [Auerbach (130)], somatotropic hormone from porcine pituitaries [Ottaway (131)], and thyroid hormones [Galton & Pitt-Rivers (132)]. The Endocrinology Study Section of the National Institutes of Health (Bethesda 14, Maryland, U.S.A.) has made available follicle stimulating hormone in vials of 5 or 25 mg. for research purposes [Hill (133)].

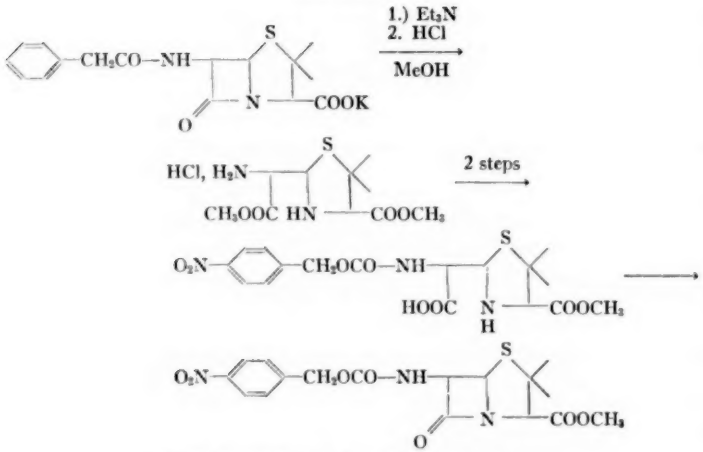
Various peptides.—Peptides obtained by degradation of proteins and exhibiting growth-promoting properties for *Lactobacillus casei* and *Streptococcus hemolyticus* (strepogenin) have been studied by Mikes, Schuh & Šorm (134) and by Mikeš & Šorm (135). Since peptides of the most diversified structure elicit similar effects, it is concluded that strepogenin is not a chemical, but a purely biological, appellation; however, the presence of glutamine and asparagine seems to be essential. Additional peptides that have been isolated from natural sources are: arginyl-glutamine from *Cladophora* species [Makisumi (136)], γ-glutamyl-S-methyl-cysteine from *Phaseolus vulgaris* [Zacharius, Morris & Thompson (137)], and S-sulpho-glutathione from lens of calves [Waley (138)]; the synthesis of the S-sulpho-glutathione was accomplished by reacting the disulphide of glutathione with disodium sulphite. In spite of observations to the contrary [Chen (139)], the presence of a sexually specific peptide in males of *Drosophila melanogaster* Meigen (16 genotypes were tested) has again been demonstrated [Fox, Mead & Munyon (140)].

SYNTHETIC ANALOGUES AND DERIVATIVES

Following the recognition of the biological, pharmacological, and therapeutical possibilities inherent in many amino acids and peptides, attempts have been made to obtain, by synthesis, analogues of such compounds that might display qualitatively or quantitatively different biological activities or even antagonize the natural compounds.

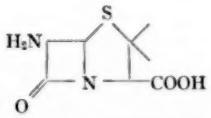
Peptides

Antibiotics.—Sheehan & Ferris (141) have developed a method (XXI) for the partial synthesis of analogues of penicillin; the synthesis starts with penicillin G.



XXI. Partial Synthesis of Penicillin Analogues

An even more promising natural starting material, 6-amino penicillanic acid (XXII), from which such analogues might easily be prepared, has been found by Batchelor and co-workers (142), in cultures of *Penicillium chrysogenum* W.51.20.

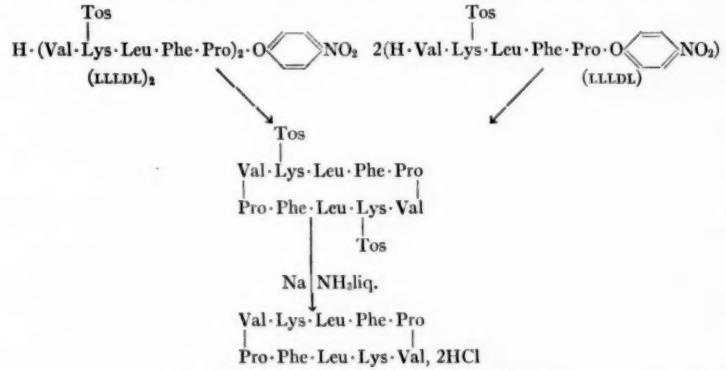


XXII. 6-Amino penicillanic acid.

Erlanger, Curran & Kokowski, in continuation of their study on open-chain peptides related to gramicidin S, have prepared two peptide intermediates containing L-phenylalanine in place of the D-isomer[5] (143): Z·Val·Orn(Tos)·Leu·Phe·Pro·OMe (L5) and H·Pro·Val·Orn(Tos)·Leu·Phe·Pro·OCH_3, HCl (L6), as well as the decapeptide containing L-lysine residues in place of L-ornithine (144). Schwyzer & Sieber (50, 145), working along similar lines in the cyclic series, have prepared the cyclic analogue of gramicidin S, which contains two lysine residues in place of ornithine. The synthesis is illustrated in sequence XXIII.

The nicely crystalline compound exhibits antibiotic effects, *in vitro*, which are quantitatively similar to those of gramicidin S, thus demonstrating that the unnatural amino acid ornithine is not directly responsible for the antibiotic properties.

Hormones and other peptides.—In the oxytocin series, Phe^2-oxytocin has been prepared almost simultaneously by Jaquenoud & Boissonnas (146) and by Bodanszky & du Vigneaud (147). Biological comparison with oxytocin shows that the phenolic hydroxyl contributes strongly to the activity but



XXIII. Synthesis of Gramicidin-S Analogues

that it is not essential. Isoasparagine oxytocin, the isoasparagine isomer of oxytocin [Lutz *et al.* (148)], like the isoglutamine isomer, is biologically almost inactive. This indicates that the size of the heterodetic ring, or the presence of normal peptide bonds, or both are determining factors.

In our laboratories, a number of analogues of hypertensin I and of hypertensin II (angiotensin) have been prepared and partly published [Schwyzer and co-workers (149); Gross (150); Schwyzer (151, 152)]. Their effects on the blood pressure of nephrectomized rats may be summarized as follows: amide groups on the β-carboxyl of Asp[1] do not lower the potency, whereas an amide group at the C-terminal end is detrimental; changes in the amino acid sequence generally lower the potency in a manner not yet completely understood; and omission of Asp[1] or its replacement by Gly in hypertensin II leaves this biologic property almost unaltered.

King, Clark-Lewis & Swindin (153) have synthesized a dimethyl analogue of glutathione that contains D,L-penicillamine in place of cysteine; phthaloyl-glutamic anhydride was used to establish the γ-glutamyl bond. Results of the biological comparison of this "penithione" with the natural product are being awaited with interest.

Amino Acids

A very great number of amino acid analogues have been synthesized during the period under review. The term "analogue" is used here in a wide sense to include also certain types of derivatives. Space forbids even a brief account of the more important publications. Much of the work was carried out in the hope of finding antitumor or antibacterial agents. Other authors were concerned with the more basic problem of antimetabolism to amino acids and of enzyme inhibition by substrate analogues. Gradually, work is emerging which indicates that more general pharmacological effects may be caused by such compounds. With this development, the field of amino acid analogues is slowly merging into that of synthetic pharmaceuticals.

Antibiotic, antitumorous, and related activities.—Amino acid analogues and derivatives which possess antibiotic, antitumorous and related activities include: antibacterial esters of L-tryptophan [Perry, Kaplan & Stock (154)]; cycloserine and related compounds [Kochetkov *et al.* (155); Khomutov *et al.* (156, 157)], 5-diazo-4-oxo-L-norvaline [Liwschitz, Irsay & Vincze (158)]; N-substituted glutamines [Edelson, Skinner & Shive (159)]; cysteine thioethers from chloroethylenes [McKinney, Eldridge & Cowan (160)]; O-[N-di-(2-chloroethyl)-carbamoyl]-serine and -threonine [Bergel & Wade (161)]; derivatives of *p*-amino- and *p*-mercapto-phenylalanine [Bergel & Stock (162)]; N′-α-aminoacyl-NN-di(2-chlorethyl)-*p*-phenylenediamines [Bergel & Stock (163)]; α-hydroxylamino and α-oximino acids [Wilson and co-workers (164)]; derivatives of cysteine, homocysteine, and cysteamine [Goodman, Ross & Baker (165)]; and N-(6-purinyl)-amino acids [Osdene & Ward, (166)].

Antimetabolites and enzyme-inhibitors.—Antimetabolites and enzyme-inhibitors include: cyclohexen-3-yl-l-D,L-alanine, an analogue of leucine [Edelson *et al.* (167)]; D,L,-β-2-fluorenyl alanine [Morrison (168)]. The interaction of α-chymotrypsin with α-N-carbethoxy-D- and L-tyrosin-methylamide was investigated by Manning & Niemann (169).

Other biological properties.—N-tosylamino-acids and their N-alkyl derivatives as synthetic antidiabetic compounds [Budesinsky *et al.* (170)]; N-acetyl-5-hydroxy-tryptophan and related compounds [Koo, Avakian & Martin (171)]; and 5-bromotryptophan and derivatives [Harvey (172)].

Additional papers.—Subjects of other papers that have appeared during the first half of 1959 on amino acid and peptide isolation and determination include the following: reaction of proteins with amino acid thiophenyl esters, and an ultramicro procedure for Edman's degradation of radioactive peptides (173); paper chromatography of dinitrophenyl amino acids, a review (174); dinitrophenylene bisamino acids from cysteine, glycine, lysine, and tyrosine (175); separation of dinitrophenyl amino acids and ether-soluble dinitrophenyl amino acids from wool (176); column-chromatographic separation of dinitrophenyl amino acids and dinitrophenyl peptides on polyamides (177); improved method for paper-chromatographic separation of dinitrophenyl amino alcohols (178); chromatography of neutral amino acids on cellulose columns with ethanol-water mixtures (179); paper chromatography and R_F-values of amino acids and other organic compounds in selected solvents (180); separation on a preparative scale of amino acids from a protein hydrolysate by use of ion-exchange resins (181); continuous separation of amino acids on an ion-exchange resin column (Dowex 50 X*8*) by gradient elution with citrate buffer (182); quantitative determination of amino acids in mixtures by automatic recording (183) after high tension electrophoresis; rapid quantitative determination of amino acids by paper chromatography at 60° and reading of maximum color density after treatment with triketohydrindene hydrate (ninhydrin) (184); spectrophotometric determination of amino acids (alkaline copper-salt method using cuprizone and biscyclohexanone oxalyldihydrazone) (185); analysis of mixtures of amino acids by gas-phase chromatography (186); rapid quantitative determination of ar-

ginine, histidine, and lysine by ion-exchange paper (cellulose+Amberlite XE-69) chromatography (187); microchemical determination of histidine in protein hydrolysates and urine (188); rapid quantitative horizontal paper chromatography at elevated temperatures—for the determination of aspartic and glutamic acids (189); new color reaction for tryptophan and tryptophan-containing peptides (190); estimation of angiotensin in blood (191); and chromatographic separation of oxytocin and vasopressin on carboxymethyl cellulose (192).

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METABOLISM OF AMINO ACIDS[1,2]

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Introduction

This review is limited to amino acid metabolism and does not include those aspects of amino acid biochemistry related to activation, protein synthesis, proteolysis, transport, nutrition, or chemical structure; these topics are discussed elsewhere in this volume. The last section of this review concerns amino acid metabolism in various disease states. Although developments in all areas of amino acid metabolism have been covered, this review is not intended as a summary of all papers relating to this subject. It will also be obvious to the reader that certain areas have received special emphasis; this is a reflection of the interests of the authors.

Glycine and Serine

With respect to the mechanism of glycine formation, although isocitric lyase, or isocitritase, which catalyzes the reversible cleavage of L$_s$-isocitrate to glyoxylate and succinate, is present in molds, isotopic studies showed (1) that the formation of glycine from citrate was not of major importance in molds.

Stadtman (2) found that a quinone was required in the glycine reductase system of *Clostridium sticklandii*. The partial loss in reductase activity by lipid solvent extraction could be reversed by the addition of D-α-tocopherol but not of vitamins K_1 or K_2. Dajani & Orten (3) showed that glycine could be utilized by nucleated erythrocytes for synthesis of the individual Krebs-cycle acids. However, the mechanism for incorporation of glycine into the citric acid cycle intermediates was left unexplained.

Accumulated evidence has indicated an origin of deoxyribose distinct from that of ribose and involving condensation of a 2-carbon unit with a 3-carbon unit. Shreeve (4), using partially hepatectomized rats, showed that 2-C^{14}-glycine did not specifically label carbon-5 of deoxyribose and thereby eliminated δ-aminolevulinic acid (or a similar compound) as a precursor of deoxyribose.

[1] The survey of the literature pertaining to this review was concluded in October, 1959.

[2] The following abbreviations are used in this chapter: CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); TPNH for triphophopyridine nucleotide (reduced form).

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Shuster & Goldin (5) showed that administration of high doses of nicotinamide to mice stimulates the incorporation of formate and glycine into the acid-soluble adenine of the liver but not into liver protein. They conclude that nicotinamide stimulates the net synthesis of the adenine moiety required for the formation of new DPN. In confirmation of previous studies with glycine-N^{15}, the initial incorporation of glycine-1, or 2-C^{14}, or glycine-N^{15} into uric acid was significantly greater in gouty hyperexcretors than in normal subjects, whereas the patterns of incorporation in gouty normal excretors was indistinguishable from that of normals (6).

The conversion of glycine to serine, a reaction which is widespread in nature, was demonstrated in two other plants (7, 8) and in silk-worms (9). A soluble mitochondrial dimethylglycine dehydrogenase, distinct from sarcosine oxidase, was prepared by Knowles & Sakami (10). The enzyme converts dimethylglycine to formaldehyde and sarcosine in the presence of phenazine methosulfate. Wriston & Al-Shukri (11) showed that folic acid and pyridoxalphosphate are involved in the conversion of sarcosine to serine but do not participate in the oxidation of sarcosine. Pyridine nucleotide is also implicated in sarcosine metabolism, since the injection of 6-aminonicotinamide into animals resulted in a diminished mitochondrial oxidation of sarcosine.

Hübscher *et al.* (12) showed that the biosynthetic pathway of phosphatidyl serine differs from that of phosphatidyl choline or of ethanolamine. In preparations of rat liver mitochondria, the cofactor requirements were found to be cytidine-monophosphate, ATP*, CoA, and Mg^{++}. Cytidine-triphosphate, which is required for the synthesis of phosphatidyl choline, was found to be inhibitory in this system.

The identification of D-serine in lombricine (guanidinoethyl-seryl-phosphate) by Beatty *et al.* (13) establishes, for the first time, the existence of a D-amino acid in animal tissues. Evidence was presented for different mechanisms whereby L- and D-serine are converted to glucose (14).

Neuhaus & Byrne (15) purified O-phosphoserine phosphatase from chicken liver acetone powder. The enzyme catalyzes the following reactions:

$$\text{P-serine} \xrightarrow{Mg^{++}} \text{serine} + P_i$$

$$\text{P-serine} + \text{serine-}C^{14} \xrightarrow{Mg^{++}} \text{P-serine-}C^{14} + \text{serine}$$

No evidence for a serine kinase that utilizes ATP could be found. The only active substrates for the phosphatase were phospho-L- and -D-serine and *p*-nitrophenyl phosphate. However, evidence was presented to show that a contamination of O-phosphoserine phosphatase by an uncharacterized phosphatase was responsible for the hydrolysis of the nitrophenyl phosphate. Borkenhagen & Kennedy (16) purified a similar enzyme from rat liver. In agreement with the previous workers, they showed that the enzyme has a much higher affinity for phospho-L-serine than for phospho-D-serine and that both substrates are hydrolyzed at the same active site on the enzyme.

Miyaki *et al.* (17) reported that *Proteus morganii* decomposes ethanol-

amine to ammonia and acetaldehyde. The methylated ethanolamines were found to be inert. A probable mechanism for deamination was offered on the basis of isotopic studies. A cholineless mutant of *Neurospora crassa* was shown to accumulate free mono-, di-, and trimethyl ethanolamines in decreasing order (18). Bremer & Greenberg (19) reported that the phospholipids in the livers of rats injected with methyl-C^{14} methionine contained labeled mono- and dimethylethanolamine as well as choline. They propose that the methyl acceptor in choline synthesis is the fully formed cephalin molecule.

Alanine

Although reductive amination of pyruvate has been clearly indicated for some time, an alanine dehydrogenase has not been isolated previously. Kaplanskiĭ & Berezovskaia (20) have now obtained an extract from disintegrated rat liver mitochondria which catalyzes the synthesis of alanine from ammonia and pyruvate. Glutamate-pyruvate and glutamate-oxaloacetate transaminase activities were not present in this preparation. Goldman (21) has also purified an alanine dehydrogenase from a cell-free extract of *Mycobacterium tuberculosis*. The enzyme was specific for DPN or DPNH and L-alanine or pyruvate. D-Alanine neither served as a substrate nor inhibited the reaction. On the basis of kinetic studies, it was proposed that reductive amination proceeds in two steps: the enzymic formation of enzyme-bound imino acid from pyruvate, followed by the enzymic reduction of the imino acid by DPNH to the amino acid.

Ikawa & Snell (22) subjected *Streptococcus faecalis R* cells, grown with D-alanine-1-C^{14}, to trichloroacetic acid extraction; they found 10 per cent of the radioactivity in the cold acid extract, 37 per cent in the hot acid extract, and 46 per cent in the insoluble residue. D-Alanine and D-alanyl-D-alanine accounted for most of the radioactivity in the cold extract. The form in which D-alanine existed in the nondialyzable hot acid extract was not identified, but since this preparation was homogenous and was similar in composition to the cell wall, they suggested it as a material suitable for further structural investigations. Baddiley and his co-workers (23) demonstrated that trichloroacetic acid extracts of walls of several species of bacteria contained ribitol phosphate polymers (teichoic acids) having glucose residues with O-D-alanyl groups. They further demonstrated the presence of a specific D-alanine activating enzyme in bacterial extracts (24). This enzyme appears to be the first ever described for activation of a D-amino acid.

Vagelos *et al.* (25) have purified acryl CoA aminase from *Clostridium propionicum*. The enzyme was found to catalyze the amination of acryl CoA to form β-alanyl CoA. This finding not only provides a new mechanism for the synthesis of β-alanine, but also represents the first example of the enzymic formation of a CoA derivative of an amino acid. The reaction was found to be freely reversible and showed a high degree of specificity for acryl CoA and an absolute requirement for ammonia. Another pathway for the

formation of β-alanine in animal tissues had been previously described by Kupiecki & Coon (26). This involved transamination between malonic semialdehyde, derived presumably from propionic acid metabolism, and glutamate. Nishizuka *et al.* (27) purified a β-alanine pyruvate transaminase from a *Pseudomonas* species. The transamination reaction was freely reversible and pyruvate acted as the specific amino acceptor while γ-aminobutyric acid was a poorer amino donor than β-alanine.

An interesting observation on the utilization of alanine in plants was made by Shilov & Yasnikov (28). By introducing C^{14} labelled compounds through leaves and roots of a variety of dandelions, they showed that alanine-3-C^{14} and alanine-2-C^{14}, but not alanine-1-C^{14}, served as better precursors of fatty acids and caoutchouc than did labelled acetate. Alanine served almost as well as acetate as a carbon precursor for carotene. The position of the labelling was not investigated. The authors suggest that a two-carbon fragment derived through decarboxylation of alanine may be transferred or utilized in a variety of plant reactions.

Spiricher *et al.* (29) demonstrated that hog stomach acylase catalyzes the reversible synthesis and hydrolysis of L-acetylalanine. The equilibrium constant of this process was found to be 0.11.

The amino acid L(+)-2,3-diaminopropionic acid, which may be considered as amino alanine, has been shown to be present in the free state in seeds of *Mimosa Palmeri* (30); and its identity has been established beyond a doubt. This amino acid was previously found in hydrolysates of viomycin, a *Streptomyces* antibiotic (31).

Threonine

Another example of a feed-back control mechanism was demonstrated by Wormser & Pardee (32) in threonine biosynthesis. Threonine is synthesized via the pathway shown in Figure 1.

$$\text{Homoserine} \xrightarrow[\text{ATP, } Mg^{++}]{\text{kinase}} \text{homoserine phosphate} \xrightarrow{\text{mutaphosphatase}} \text{threonine.}$$

Fig. 1. Threonine biosynthesis.

It was found that homoserine kinase was inhibited competitively by L-threonine, the product of the overall series of reactions. Allothreonine and D-threonine were inactive in this respect, and other amino acids were less active than threonine. Homoserine-phosphate mutaphosphatase was not inhibited by L-threonine. This mechanism was demonstrated in yeast and in *Escherichia coli*. Flavin & Slaughter (33) purified homoserine-phosphate mutaphosphatase from a strain of *Neurospora* and found it to be partially dependent on pyridoxal phosphate for its action. Evidence was presented to eliminate a vinylglycine-like intermediate in threonine formation.

Elliot (34) isolated and identified aminoacetone from cultures of *Staphylococcus aureus* grown on a threonine or glycine-glucose medium. The mecha-

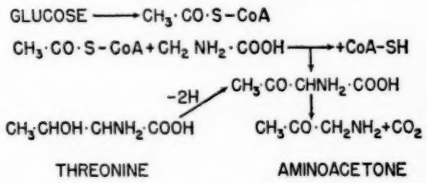

FIG. 2. Biosynthesis of aminoacetone.

nism of formation of this compound was postulated as indicated in Figure 2. This pathway is analogous to that involved in δ-aminolevulinic acid formation, except that here glycine condenses with acetyl CoA instead of succinyl CoA. The oxidation and formation of aminoacetone from glycine have been demonstrated in mammalian tissues, and the metabolic cycle illustrated by Figure 3 was suggested. This scheme provides a new route for the oxidation of threonine and an alternative to the succinate-glycine cycle for glycine metabolism. It also provides a metabolic role for the enzyme glyoxalase. Supporting evidence for the operation of such a cycle was presented by Bagchi & Guha (35). When acetate-1-C^{14} or sodium bicarbonate-C^{14} was incubated with liver slices, the labelling in carbons 1 and 5 of glutamate accounted for only 30 to 46 per cent of the total radioactivity. The cycle above (Figure 3), in conjunction with the Krebs cycle, could serve to randomize the labelling.

GLUTAMATE AND ASPARTATE

The unique decomposition of glutamate by cell-free extracts of *Clostridium tetanomorphum*, as worked out by Barker and his colleagues, is as follows:

L-glutamate ⇌ L-threo-β-methylaspartate ⇌ mesaconate ⇌ citramalate
⇌ acetate + pyruvate

The first reaction, involving the conversion of glutamate to L-threo-β-methylaspartate, has been shown to require a coenzyme, which was identified as a

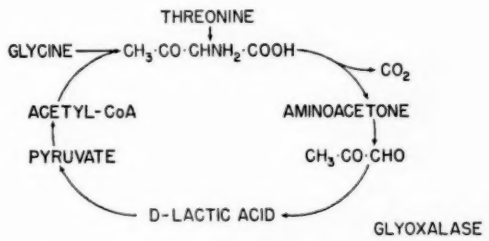

FIG. 3. Formation of aminoacetone from glycine.

derivative of pseudo vitamin B_{12} (36). Unlike the vitamin, the coenzyme contains two moles of adenine, not one. Although the organism normally makes this form of the coenzyme, it has been possible to replace the nucleotide adenine with 5,6-dimethyl benzimidazole and benzimidazole (37) by growing the organism in media containing excess amounts of these bases. The coenzyme forms of the corresponding benzimidazole vitamins also contained an adenine moiety. All three coenzymes were active in the enzymic conversion of glutamate to β-methyl aspartate, although the corresponding vitamins were inactive. The 5,6-dimethylbenzimidazole coenzyme was also present in large amounts in propionic acid bacteria and could be isolated from rabbit liver (37). The structure of the pseudo B_{12} coenzyme has not yet been elucidated, although some characteristics of the compound are known (36). The spectrum differs markedly from that of the vitamin, and the active coenzyme is readily inactivated by visible light and cyanide. The inactivation, in each case, is accompanied by a marked change in the spectrum of the coenzyme which yields new spectra resembling that of the vitamin. The location of the second adenine moiety appears to be on the cobalto-corrin structure, possibly in the position normally occupied by cyanide in the vitamin. It is evident from this work that a new class of B_{12} compounds exists in nature, and these results may help to elucidate the various roles in which vitamin B_{12} has been implicated (38 to 44).

The enzyme that catalyzes the reversible conversion of L-threo-β-methylaspartate to mesaconate has been purified and studied by Barker *et al.* (45). Aspartate, which undergoes a similar reaction, is only 1/100 as active as β-methylaspartate in this system. The L-threo isomer is the normal substrate, although weak activity was seen with the L-erythro compound. A definite requirement for K^+ was found. The L-threo isomer was enzymically prepared, isolated, and characterized by Barker *et al.* (46). Benoiton *et al.* (47) synthesized DL-β-methylaspartate by chemical procedures but only obtained one of the two possible diastereomeric racemates. The D and L isomers were separated with renal acylase II.

Sallach (48) has studied a metabolic pathway of hydroxyaspartic acid in preparations from Ehrlich's ascites carcinoma and rat liver. He was able to demonstrate the following transcarbamylation reaction:

$$\text{Carbamyl-PO}_4 + \text{hydroxyaspartic acid} \rightarrow \text{N-carbamyl hydroxyaspartate.}$$

Goldstein (49) has demonstrated enzymic formation of N-acetyl aspartate from acetyl CoA and L-aspartate. Using a partially purified extract of rat brain (50), it was shown that various univalent inorganic anions stimulated the reaction, while a number of fatty acid CoA derivatives were competitive inhibitors.

Bagchi & Guha (35) found that rat liver incorporates acetate-C^{14} and $HC^{14}O_3^-$ into glutamate. However, the major portion of the labelling was not in carbon atoms 1 and 5 of glutamate. The high labelling in the other carbon atoms could be explained by a pathway via pyruvate-2-C^{14}.

Wilson & Koeppe (51), attempting to explain earlier results, have found marked differences, depending on the site of injection, in the manner in which glutamate is metabolized in animals. DL-Glutamate-2-C^{14} was injected either intraperitoneally, into the cecum, or administered by stomach tube. After intraperitoneal injection, the major pathway involved conversion to succinate via α-keto-glutarate. Following injection into the cecum, the C_2 of glutamate appeared as the methyl group of acetate. Glutamate was metabolized by both pathways when administered by stomach tube. This is another example of the difficulties, attributable to intestinal tract bacteria, involved in animal experiments, *in vivo*.

In the past year, various studies have appeared on the inhibition of glutamic acid dehydrogenase. Inagaki (52) has studied the inhibitory affects of urea and a bacterial protein preparation. Urea, at low concentrations, competed with the substrate and acted as a reversible inhibitor; both DPN and glutamate protected the enzyme, while DPNH was found to accelerate the inhibition. Many metal-complexing agents, as well as silver ion and mercury compounds of the type R-Hg-X, have been shown to inhibit glutamic acid dehydrogenase (53). In the study by Hellerman *et al.* (54), the calf liver enzyme was shown to contain three sulfhydryl groups per protein subunit. Sulfhydryl groups were determined by measuring changes in enzyme activity during titration of the enzyme protein with the inhibitors mentioned above.

An excellent study by Frieden (55, 56) on the effect of coenzyme on the sedimentation velocity, kinetic behavior, and association-dissociation properties of glutamic dehydrogenase may help to explain the manner by which the adenosine nucleotides and other compounds produce inhibition. DPN, TPN, and their reduced forms, in concentrations used for the determination of the kinetic constants, influenced the sedimentation behavior of the enzyme. The rate of the enzymic reaction was determined by the degree of association or dissociation of the enzyme protein. When dissociated, the enzyme was inactive. At concentrations lower than $3\times10^{-4}M$, the presence of coenzyme favored association of the enzyme subunits. The active site on the enzyme protein was the same for all the coenzymes, although DPN and DPNH were also bound to a second non-catalytically active site. Binding of the adenosine nucleotides also took place at this non-active site on the enzyme. In general, compounds that affected the overall velocity influenced the dissociation or association of the enzyme molecule. The effects observed were dependent on the coenzyme used. The inhibition data presented could thus be interpreted by the specificity of the active and non-active site on the enzyme and by the effect of the coenzyme on the dissociation-association behavior of the enzyme.

Miscellaneous observations.—Crystalline liver-glutamic-dehydrogenase has been shown to catalyze the conversion of α-keto valeric acid to L-norvaline (57). The normal beating heart was found to metabolize glutamate-C^{14}; 22 per cent and 3.6 per cent were recovered as CO_2 and glutamine, respectively, within 1 hr. (58).

Miyaki *et al.* (59) have further verified early studies by Umbreit & Heneage (60) that β-hydroxy glutamic decarboxylase of *E. coli* differs from glutamic decarboxylase. The activities of the two enzymes were found to vary during the growth cycles, and β-hydroxy glutamic decarboxylase was more effectively inhibited by carbonyl reagents. It appears, however, that glutamate induces the formation of both enzymes.

Glutamine, Asparagine, and Ammonia

Studies concerning the metal requirements of glutamine synthetase have been reported by Greenberg & Lichtenstein (61). By use of purified sheep brain extracts, they have shown that with high concentrations of Mn^{++}, the pH optimum was 5.5, comparable to that required for glutamotransferase activity. On lowering the Mn^{++} concentration, the pH optima of both glutaminase and glutamotransferase were shifted towards the alkaline region. Using Mg^{++}, the pH optimum for both reactions was 7.2. These findings are consistent with the current views that both reactions are catalyzed by the same enzyme. Ting-Seng (62) has reported detailed studies on the mechanism of formation of glutamine from asparagine and glutamic acid in animal tissues. He found no evidence for the occurrence of a direct amide transfer from asparagine to glutamic acid.

As for the metabolism of glutamine, Sayre & Roberts (63) have described a simple procedure for the preparation from dog kidney cortex of a soluble glutaminase purified 100- to 400-fold. As a result of studies with enzyme inhibitors, ammonia, structural analogues, and models, they suggested that glutaminase has one sulfhydryl and two cationic sites. One of the cationic sites may serve as a point of attachment for phosphate ion, with the substrate attaching to the other cationic site through the α-carboxylate group and to the second negative charge of the phosphate through a positively charged α-amino group. Valuable thermodynamic data for glutamine and asparagine, including ΔH and ΔF for the enzymic hydrolyses of the amidine groups, have been presented (64, 65).

An interesting report concerning the effect of barbiturates on brain asparaginase in rats was presented by Miloslavskaia (66). Asparaginase activity decreased sharply and inhibition was almost complete with more prolonged sleep. By contrast, glutaminase activity was altered very little. The effects of factors such as drugs (67), anoxia (68), and bacterial toxins (69) on the content of glutamine and ammonia in rat brain have also been reported. Previous reports (70) concerning the presence of appreciable amounts of ammonia in allantoic fluid have now (71) been explained by the demonstration of considerable glutaminase activity in foetal membranes and kidneys. Pasieka & Morgan (72) have presented further evidence that cell strains of predominantly malignant origin, in tissue-culture, reduce the glutamine content of the medium; this behavior is in contrast to that of non-malignant cells, which, in many cases, even increase the glutamine content. This would be more convincing were it not for the fact that all the malignant tissues were

of human origin and almost all of the non-malignant ones of animal origin.

Without very good evidence, Martinson *et al.* (73) have again introduced the problem of gastric urease, questioning its bacterial origin and suggesting an important physiological function. This, they say, is in accord with the views of Conway (74) but in contradiction to those expressed by Kornberg & Davies (75).

γ-Aminobutyric Acid

γ-Aminobutyric acid is widely distributed in nature, being found in plants, animals, and bacteria. In mammals, it is formed from glutamate by decarboxylation, a reaction which is confined largely to the central nervous system (76). The finding of γ-aminobutyric acid in brain was not accorded any great significance until it was reported to be the main constituent (77) of Florey's neurohumoral inhibitory Factor I (78). Since then, interest in the metabolism of the compound, particularly in the central nervous system, has been intensified. There have been many reports concerning its actions on a variety of peripheral and central physiological systems both *in vitro* and *in vivo* (79 to 83). Differential effects of γ-aminobutyric acid on brain and muscle have also been reported (84). There is little doubt that the concentration of this compound is much higher in brain than in other tissues, and, as a result, suggestions have been made implicating it as a neurohormone (85, 86). However, identification of Factor I with γ-aminobutyric acid has been questioned (87, 88, 89). Furthermore, the high concentrations of this acid in the grey matter of the central nervous system (>100 mg. per cent) and its participation in active, energy-yielding metabolism make it likely that this compound functions in more ways than as a neurohumoral agent.

The implication of γ-aminobutyric acid in neuronal function has prompted studies on the metabolism of this compound in nervous tissue. As a result, many systems involved in γ-aminobutyric acid metabolism have been studied more intensively, and new routes have been suggested, some of these leading to compounds which may be even more active physiologically than γ-aminobutyric acid itself.

It has been shown that administration of hydrazides lowers its concentration in the central nervous system by inhibiting glutamic acid decarboxylase *in vivo* and that this can be reversed by the administration of vitamin B_6 (85, 90). The epileptiform seizures produced by the hydrazides have been considered to result specifically from a lowering of γ-aminobutyric acid levels in the brain. Toxopyrimidine, another convulsant agent and a vitamin-B_6 antagonist, has also been shown to lower the γ-aminobutyric acid content of rat brain (91). Interesting speculations concerning the possible explanation of the observed convulsions in pyridoxine-dependent children by a γ-aminobutyric acid deficiency have also been presented recently (92, 93). While it is tempting to connect these convulsions of vitamin-B_6 deficiency with γ-aminobutyric acid it should be kept in mind that all amino acid decarboxylations require pyridoxal phosphate, the coenzyme form of vitamin B_6. Since

histidine, serotonin, norepinephrine, tryptamine, etc., all require vitamin B_6 for their formation, one cannot blame at the present time the effects of a deficiency of this vitamin on any one metabolite. Baxter & Roberts (94) were able to elevate the levels of brain γ-aminobutyric acid several-fold by administration of hydroxylamine to rats. They also found that, in cats, administration of hydroxylamine decreased the duration of electrically induced seizures (95). It was suggested that these effects are caused by the inhibition of γ-aminobutyric acid transamination.

It has been shown that γ-aminobutyric acid is not randomly distributed in brain but is highly concentrated in the gray matter (96) of specific areas. Continuation of such studies on localization of the compound may provide a clue as to its physiologic role. It is of interest that in amphibians, too, this acid is found in high concentrations in brain (97). Albers & Brady (98) have now shown that, in the brain of the Rhesus monkey, the enzyme glutamic acid decarboxylase is also localized in cortical gray matter. In these studies, the procedure used for assaying the enzyme involved a microradiometric method with L-glutamate-U-C^{14} as substrate; by this method one can detect as little as 10^{-11} moles of CO_2 with samples of brain tissue of 3 to 15 μg.

Not only is γ-aminobutyric acid and the enzyme involved in its synthesis localized in the gray matter of the brain, but so is the enzyme responsible for the major route of metabolism of this substance—γ-aminobutyric acid-α-ketoglutarate transaminase (99). There are, however, some important differences in the local concentrations of the two enzymes. The authors believe these differences to be significant and to favor a homeostatic function for γ-aminobutyric acid rather than a neurohumoral function. The procedure used for assaying the transaminase, involved the measurement of succinic semialdehyde by condensation of the aldehyde with 3,5-diaminobenzoic acid. The resulting product was highly fluorescent and could be assayed in the spectrophotofluorometer (activation, 405 mμ; fluorescence, 505 mμ). Baxter & Roberts (100) have reported further studies on the γ-aminobutyric acid-α-ketoglutarate transaminase from beef brain. By use of acetone powder extracts they were able to demonstrate a pyridoxal phosphate requirement. Other factors, such as pH optimum, substrate stabilization and specificity, and the effects of various inhibitors, were also studied. A copper chelation method for measuring glutamic acid formation by the enzyme was also described.

The product formed by γ-aminobutyric acid transaminase, succinic semialdehyde, is an excellent substrate of aldehyde dehydrogenase (101) and is, therefore, readily converted to succinic acid, which can be metabolized in the usual manner. Studies by McKhann & Tower (102) afford additional evidence that, in the cerebral cortex, γ-aminobutryic acid and succinic semialdehyde can function as oxidative substrates almost as well as glucose and glutamate. They found that with γ-aminobutyric acid as substrate, P/O ratios of about three were obtained, indicating that the compound can be utilized as an effective energy source. Similar values were obtained with pyruvate and glutamate. As a result of these findings and similar findings by

others, these authors raise the question which many in the field have continually raised: "Does γ-aminobutyric acid in brain serve mainly in a neurohumoral type of role?" Wilson, Hill & Koeppe (103) have reported that, following administration of γ-aminobutyric acid-4-C^{14} to rats, the labelling patterns observed in glutamate, aspartate, alanine, and glycogen, isolated from tissues, are such as to suggest that all the catabolism of the substance occurs through the succinate pathway. These findings are in agreement with currently accepted concepts concerning metabolism of this substance in animals.

There have been some claims, however, that γ-aminobutyric acid metabolism in brain proceeds mainly by oxidative pathways which do not involve transamination (104). One oxidative pathway of γ-aminobutyric acid metabolism, which was first suggested a short time ago (105, 106), involves oxidation to γ-amino-β-hydroxybutyric acid. Since the latter compound also inhibits nerve transmission in a manner similar to Florey's Factor I (107) and inhibits seizures (108), additional interest has been aroused by it. Ohara *et al.* (109) have reported the isolation of β-hydroxy-γ-aminobutyric acid in crystalline form from brains of a number of animal species, including man. By use of a colorimetric procedure (the details of which were not given), they estimated the β-hydroxy-γ-aminobutyric acid content of wet temporal lobe of cattle to be 48.6 mg. per 100 gm. Unfortunately, none of these biochemical findings (105, 106, 109) have as yet been corroborated in other laboratories.

Although the presence of β-hydroxy-γ-aminobutyric acid in animal tissues remains to be substantiated, the corresponding betaine, carnitine, has been known to occur in tissues, where it is found in largest amounts in muscle. Its exact function is still undetermined, and reports concerning effects on various physiologic processes appear from time to time. Fritz & McEwen (110) have reported on the effects of carnitine on fatty acid oxidation by muscle. A comparison of the effects of carnitine and other substances on nerve conduction has also appeared (111). Pharmacological studies with γ-butyrobetaine, the quaternary ammonium derivative of γ-aminobutyric acid, have been carried out (112), and it was reported that γ-butyrobetaine appeared in the brains of mammals in which convulsions were induced by the intraperitoneal injection of cerebral convulsants.

A route of metabolism of γ-aminobutyric acid, of recent interest, involves conversion to γ-guanidinobutyric acid. The latter compound, which has been known for some time to be present in invertebrate tissues (113), was recently reported to be present in brain (114). Following this report, Pisano *et al.* (115) were able to demonstrate that γ-aminobutyric acid can accept an amidine group from arginine in the presence of mammalian kidney transamidinase. The activity with the compound was approximately one-seventh that with glycine, and, except for slight activity with β-alanine, few other substrates had appreciable activity. More recently, Irreverre & Evans (116) have been able to isolate γ-guanidinobutyric acid from calf brain in sufficient quantity to establish its identification beyond a doubt.

In the hope that some form of γ-aminobutyric acid in brain will prove to

have even more potent action as a humoral agent, many derivatives have been investigated. One such compound, γ-aminobutyrylcholine, produced interesting electrical changes in the cerebral cortex (117). Details concerning the synthesis of this compound and some of its pharmacological properties were also presented. Although it has been suggested that its effects on electrical activity are similar to those produced by various physiological actions, evidence for its presence in tissues and its formation there is still wanting.

γ-Aminobutyric acid has long been known to be present in plant material. Recently, Macpherson & Slater (118) have reported on its formation in silage and microbe-free grass. Under conditions which were unfavorable for the formation of glutamine, a large proportion of glutamic acid was converted to γ-aminobutyric acid. Kretovich & Galyas (119), utilizing wheat and barley sprouts, reported a large consumption of γ-aminobutyric acid during the utilization of oxaloacetic acid. Since the product formed, in this case, was aspartic acid, they concluded that γ-aminobutyric acid plays an important role in transamination processes in plants.

A most significant development in the area of γ-aminobutyric acid metabolism was made by Scott & Jakoby (120) when they obtained a strain of *Pseudomonas*, through enrichment culture technique, that could use pyrrolidine as the sole source of nitrogen. The following reaction sequence was demonstrated:

pyrrolidine → γ-aminobutyric acid → succinic semialdehyde → succinate.

Of great importance were the findings that γ-aminobutyric acid-α-ketoglutarate transaminase and succinic semialdehyde dehydrogenase were highly specific and could be readily obtained in purified and soluble form from this bacterial source. Detailed studies on the two enzymes have now been reported by these authors. By use of standard procedures for enzyme purification, they were able to purify the bacterial transaminase (121) to a specific activity of 3.4 μM per min. per mg. of protein. With this preparation, they were able to determine equilibrium constants for the reaction and to study the kinetics of the reaction in some detail. Although they were not able to demonstrate a direct requirement for pyridoxal phosphate, carbonyl reagents did inhibit the reaction. With respect to specificity, negative results were obtained when the following substitutions were made: β-alanine, δ-aminovaleric acid, ε-aminocaproic acid, ω-aminocaprylic acid, ornithine, or lysine for γ-aminobutyric acid; pyruvic acid, oxaloacetic acid, ketomalonic acid, or α-ketoisolvaleric acid for α-ketoglutaric acid; aspartic acid, L-2,4-diaminoglutaric acid, alanine, or D-glutamic acid for L-glutamic acid; glyoxylic acid, glycolaldehyde, or malonic semialdehyde for succinic semialdehyde.

The succinic semialdehyde dehydrogenase (122), purified to approximately the same extent, was also studied in detail. It was found to be highly specific and did not act on glyoxalate, malonic semialdehyde, and glyceraldehyde-3-phosphate. Because of the marked specificity and high activity of these two enzymes, Jakoby & Scott (122) were able to devise a most sensi-

tive and specific procedure for the assay of either γ-aminobutyric acid or α-ketoglutarate by coupling the two. The net result of the coupled reaction is shown below:

$$\gamma\text{-aminobutyric acid} + \alpha\text{-ketoglutarate} + TPN^{+} + H_2O \rightarrow \text{succinate} + \text{glutamate} + TPNH$$

The assay can be carried out with a preparation that does not require extensive purification and can be used to determine microgram quantities of γ-aminobutyric acid with high precision. This is certainly the method of choice for measuring the content of the compound in tissue extracts. A report of its application in this manner has already appeared from another laboratory (97). Another bacterial source of γ-aminobutyric acid-α-ketoglutarate transaminase has also been reported (123).

Lysine

Lysine is one of the few amino acids for which the biosynthetic pathway in bacteria is different from that found in other organisms. In most bacteria (Gram-positive cocci excepted) and in blue-green algae, α-ε-diamino-pimelic acid occurs, presumably as a precursor of lysine and as a component of the cell wall. Until recently, the only information available concerning the biosynthesis of α-ε-diamino-pimelic acid came from studies of Roberts *et al.* (124) which indicated that aspartic acid could serve as a precursor of four of the seven carbon atoms of this compound. The presence of a racemase capable of interconverting meso- and L-α-ε-diamino-pimelic acid (125) may also be of significance in the biosynthetic process. Further information on the biosynthesis of the compound is now available. Gilvarg (126) was able to demonstrate that, in both resting cells of *E. coli* and in cell-free extracts, not only aspartic acid-C^{14}, but also pyruvic acid-C^{14}, could serve as efficient sources of α-ε-diamino-pimelic acid. Thus, it would appear that aspartic acid is a precursor of four of the carbon atoms and pyruvic acid a source of the other three. Gilvarg (126) was also able to obtain appreciable formation of α-ε-diamino-pimelic acid in dialyzed cell-free extracts of *E. coli*. With this preparation, he demonstrated requirements for ATP, pyridine nucleotide, and Mg^{++}, which suggested activation of either pyruvate or aspartate, a condensation, and a reduction to the oxidation level of α-ε-diamino-pimelic acid. Stimulation of this reaction by glutamate suggested that the glutamate may supply the second amino group as a source of the succinyl side chain leading to N-succinyl-L-α-ε-diamino-pimelic acid. A specific N-succinyl-diaminopimelate-glutamate transaminase was purified from another strain of *E. coli* (127). N-Succinyl-L-α-ε-diamino-pimelic acid, which was originally suggested as an intermediate and previously found to be nutritionally inactive (128), was readily converted to α-ε-diamino-pimelic acid by this cell-free system. Meadow & Work (129), also using *E. coli*, found that the diamino-pimelic acid excreted into the medium under conditions of lysine-restriction was not always derived from the same precursors as the intra-

cellular α-ε-diamino-pimelic acid. In a second paper (130), utilizing other strains of *E. coli* and C^{14} substrates, these authors presented evidence indicating that this substance is an important, but not the sole, precursor of lysine in bacteria. They were also able to demonstrate the virtual irreversibility of its decarboxylation. Problems of penetration of various precursors and intermediates into the bacterial cells were also discussed in an attempt to explain previous reports which were contrary to their conclusions that α-ε-diamino-pimelic acid is on the direct biosynthetic route to lysine. Evidence that ketoadipic acid and α-amino-adipic acid may be precursors of lysine in yeasts and molds was also presented (131, 132).

An interesting finding of a lysine racemase in preparations of a strain of *Proteus vulgaris* (133) has now been extended (134) to indicate that this activity is widely distributed in the genera *Proteus* and *Escherichia*. By use of autolysates of *Proteus vulgaris*, it was shown that neither glutamic acid nor alanine was appreciably racemized, thus making it highly unlikely that this racemization is composed of several transamination steps. This enzyme has also been found in a strain of *Pseudomonas* (135). It will be of interest to determine the significance of such a racemase.

The occurrence of pipecolic acid and 5-hydroxypipecolic acid in nature is well established. They arise as metabolites of lysine and hydroxylysine. 4-Hydroxypipecolic acid was reported to be found in *Acacia* (137). Fowden (138) recently isolated from *Armeria maritima* (thrift) a hydroxypipecolic acid identical with that found in *Acacia*, but showed that the material obtained from both sources was the 3- and not the 4-hydroxy derivative. Rothstein & Greenberg (139) obtained evidence that pipecolic acid was converted to glutaric acid in rats and demonstrated that rat liver mitochondria could completely oxidize pipecolic acid to CO_2.

Miscellaneous observations.—The conversion of lysine-2-C^{14} to a melanin-like substance in the feathers of turkey poults was demonstrated (136). *Salmonella typhimurium* was reported to convert lysine to ε-N-methyllysine and to incorporate the methyl amino acid into flagella proteins (140). Mechanic & Levy (141) showed that a hydrolyzate of collagen from Achilles tendon contained a tripeptide of glycine, glutamate, and lysine (peptide-bonded at the ε-amino group).

Leucine, Isoleucine, and Valine

It is customary to present the metabolism of these three branched chain amino acids together, not only for convenience, but because they do exhibit many points of similarity. It is now fairly well established that α-acetolactate and α-aceto-α-hydroxybutyrate are precursors in the biosynthesis of valine and isoleucine, respectively. In support of this, Wagner *et al.* (142) have been able to demonstrate the accumulation of acetylmethylcarbinol and acetylethylcarbinol by a *Neurospora crassa* mutant. These compounds, which are the decarboxylated forms of α-acetolactate and α-aceto-α-hydroxybutyrate, were formed by this mutant, presumably because of a block in the biosynthesis of the two branched amino acids. The carbinols, which appeared when the

the isoleucine and valine required for growth were almost completely gone from the medium, were identified and assayed by conversion to the corresponding pteridines by condensation with 2,4,5-triamino-6-hydroxypyrimidine. These authors also showed that decarboxylation of the aceto-hydroxy acids could occur spontaneously in an acid medium as well as by catalysis involving a decarboxylase in the mycelia. Another paper on this subject by Watanabe *et al.* (143) concerned the enzymic synthesis of L-isoleucine by cell-free preparations from bakers' yeast. These authors synthesized α-aceto-α-hydroxybutyric acid and showed that, in addition to the dialyzed yeast extract, TPN, isocitrate, Mg^{++}, and valine were required for maximal conversion to isoleucine. The first three factors support the reported requirement for TPNH in isoleucine synthesis, presumably in the conversion of the intermediate α-keto-β-hydroxy acid to the dihydroxy acid (144). The valine stimulation suggests that it may act as the amino donor in the final step of the reaction. However, no requirement for pyridoxal could be shown, and the authors did not discuss the effectiveness of other amino acids as amino donors.

As to the metabolism of these amino acids, Dehority *et al.* (145) have shown that rumen microorganisms, *in vitro*, convert a major portion of valine, first to α-keto isovaleric acid and then, by decarboxylation, to isobutyric acid. The amino acid and the acid products were found to be utilized by the microorganisms for growth and to function cellulolytically. Since the corresponding products of leucine (α-keto-isocaproic acid and isovaleric acid) and isoleucine (α-keto-β-methyl-*n*-valeric acid and α-methylbutyric acid) were also found to be cellulolytically active, it would appear that all three of the branched amino acids are metabolized in a similar manner. In *Neurospora crassa*, transamination leading to formation of valine and isoleucine from the corresponding keto acids is apparently catalyzed by one enzyme (146).

Edelson *et al.* (147) have reported the preparation of a new antimetabolite of leucine metabolism, 3-cyclohexene-1-DL-alanine. The latter compound competitively inhibited the growth of *Leuconostoc dextranicum* 8086 over a ten-fold range in concentrations of leucine.

Further studies on the utilization of leucine for carotene biosynthesis by the mold *Phycomyces blakesleeanus* were reported by Chichester *et al.* (148). This group has now completed its study of the contributions of the individual leucine carbon atoms to the carotene molecule. By use of all the possible forms of leucine-C^{14} and by degradation of the carotene to acetic acid, they have come to conclusions which are summarized in Figure 4.
Although appropriately labelled forms of β-hydroxy-β-methyl glutarate and mevalonate (postulated intermediates) were incorporated into carotene, their isotopic dilutions did not agree with values calculated from the leucine data. The authors suggest that C-4 of leucine may recycle with an active acetoacetate pool.

Ferguson *et al.* (149) have demonstrated conversion of the leucine metabolite β-hydroxy-β-methyl glutaric acid to mevalonic acid by yeast extracts and have studied some of the requirements for this conversion.

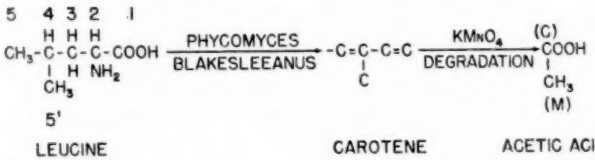

FIG. 4. Derivation of carotene from leucine. The carboxyl carbon (c) of acetic acid, shown here as a terminal oxidation product, arises from carbon atoms 2 and 4 of leucine; the methyl carbon arises from carbon atoms 3, 5, and 5′.

ARGININE, GUANIDINES, AND UREA

A most important role of arginine is its function as a precursor of creatine and the phosphagen, creatine phosphate. An excellent and highly critical review that summarizes present knowledge of the chemistry, biochemistry, and function of creatine phosphate and other phosphagens was presented by Ennor & Morrison (150). Cori *et al.* (151) have reported the presence of an enzyme, differing from both creatine phosphokinase and from acyl phosphatase, that apparently catalyzes the direct transfer of phosphate from 1,3-diphosphoglyceric acid to creatine. A report by Peterson *et al.* (152) suggested that administration of glycocyamine and betaine to rats may raise the creatine phosphate level in skeletal muscle but not in heart. Boulanger & Osteux (153) have administered uniformly labelled arginine-C^{14} to rats and have detected C^{14} in the following urinary products: arginine, ornithine, urea, creatinine, γ-guanidobutyric acid, and δ-guanido-*n*-valeric acid. The intensity of the labelling in the latter two compounds suggested to the authors that they were formed, for the most part, directly from arginine rather than by transfer of the amidine group. However, Pisano *et al.* (115) have already shown that transamidination between arginine and γ-aminobutyric acid does take place in rat tissues.

Arginine is hydrolyzed to ornithine and urea by the highly specific enzyme L-arginase. Grassmann *et al.* (154) have applied continuous paper electrophoresis in the presence of Mn^{++} to the purification of arginase. They were able, in this way, to obtain a preparation which was homogeneous in the ultracentrifuge and by electrophoresis, and free from other biological activity. A paper chromatographic method for measuring arginase in animal tissues was reported by Chorazy & Chorazy (155). In addition to L-arginase, there is known to be present in tissues a less specific enzyme which has been referred to as heteroarginase (156). Little is known about this enzyme or its significance. The D-arginase recently discussed by Nadai (157) is, in all probability, another manifestation of heteroarginase. Using D-arginine as the substrate, data were presented on the tissue distribution of the enzyme, some of its physical characteristics, inhibitors, and activators.

Growth of *E. coli* in chemically defined media did not induce formation of

arginine decarboxylase, but the addition of a casein digest resulted in enzyme formation (158). In a non-aerated culture, arginine, methionine, tyrosine, and aspartic acid could replace the casein digest, and iron was found to stimulate enzyme formation. Under aerobic conditions, the iron requirement was increased, and glutamate was also required for enzyme synthesis. No definite role of iron for enzymatic activity could be found.

A study on the effect of DL-penicillamine on various animal amino acid decarboxylases showed that arginine decarboxylase (*E. coli*) was inhibited markedly at 10^{-3} to 10^{-4} *M* (159). Metals did not reverse this inhibition.

A review by Berezov (160) of current concepts regarding mechanisms of urea synthesis has recently appeared. This is a translation of a Russian article (161) published in 1955 which, while no longer up to date, is a good review of the field to that time. Additional information has been obtained concerning some of the enzymic steps leading to urea in animal tissues. Brown & Cohen (162) have reported procedures for the estimation of five enzymes of the urea cycle which can be applied to as little as 150 mg. of liver. With these procedures, they (163) were able to investigate the urea cycle in tadpoles at the onset of metamorphosis. They showed that the arginine synthetase system was rate-limiting and that induction of all the urea-cycle enzymes occurred in a manner consistent with their *de novo* synthesis at the onset of metamorphosis. They go on to point out that this phenomenon may parallel the evolutionary development of nitrogen excretion mechanisms which are associated with land colonization.

Metzenberg *et al.* (164) investigated carbamyl phosphate synthetase from frog liver by coupling the enzyme system with the ornithine transcarbamylase reaction. The existence of two partial reactions for the former enzyme system was suggested since inorganic phosphate was released without a parallel formation of carbamyl phosphate. Furthermore, inorganic phosphate was released in the absence of added ammonium ions. All this suggests that CO_2 is activated before the participation of ammonia. The latter reaction requires the presence of N-acetyl glutamic acid.

In an earlier paper, Krebs *et al.* (165) reported a factor in washed suspensions of *E. coli* that accelerated phosphorolysis of citrulline by mammalian liver extracts. They have now been able to demonstrate that this factor is heat labile and is not a carbamyl phosphatase, although hydrolysis of carbamyl phosphate occurs readily in animal tissues and in bacteria (166). Petrack & Ratner (167) have presented further evidence which suggests a mechanism for argininosuccinic acid formation that involves concomitant cleavage of ATP to AMP and pyrophosphate. The marked stimulation by pyrophosphatase appears to be caused by inhibition of the condensing enzyme by pyrophosphate.

Ravel *et al.* (168) have purified ornithine-transcarbamylase from *Streptococcus lactis* and have studied many of its physical properties. They also found that the purified preparations contained only one part of biotin in

5,000,000 parts of enzyme. Previous reports of a biotin stimulation of ornithine-transcarbamylase activity in whole cells must, therefore, relate to an effect of biotin on enzyme synthesis.

Experiments reported by Sallach (48) suggest that aspartic transcarbamylase may not be specific since hydroxyaspartic acid can also serve as a substrate and yield carbamylhydroxyaspartic acid. If this product is metabolized in the same manner as carbamylaspartic acid, it should give rise to 5-hydroxyuridine, a known antimetabolite of nucleic acid synthesis. Murakami *et al.* (169) have injected L-carbamyl-C^{14} aspartate into mice and demonstrated incorporation into the acid-soluble pyrimidine nucleotides of brain. Their conclusion that biosynthesis of these nucleotides in brain occurs in a manner similar to that in liver and bacterial extracts is, however, not justified since preliminary metabolic changes most likely occurred in liver and other organs before incorporation into the brain nucleic acids. The possibility that the carbamyl-aspartate transcarbamylase system can operate as a regulatory mechanism for nucleic acid synthesis is suggested from experiments with normal and malignant tissues, which were reported by Calva *et al.* (170). It is of interest, too, that ornithine carbamyl transferase, which is normally found in dog serum in trace amounts, is markedly elevated (500- to 400-fold) by various procedures, including bile duct ligation and carbon tetrachloride poisoning (171).

Sulfur-Containing Amino Acids

Methionine.—Further studies on the biosynthesis of methionine in bacterial systems have appeared. Kisliuk & Woods (43) have further investigated the vitamin-B_{12} requirement that was originally reported in 1956 (42). They found that vitamin B_{12} was present in a protein-bound form, this complex being a component of the overall reaction. Hatch *et al.* (44) obtained a similar protein-vitamin-B_{12} complex, which they were able to dissociate by heat treatment. In an attempt to elucidate the nature of the one-carbon acceptor in methionine synthesis, Stevens & Sakami (172) carried out studies with an ammonium sulfate fraction of pig liver extract. Adenosylhomocysteine replaced homocysteine but did not eliminate the requirement for ATP. Although methyl methionine sulfonium homocysteine transmethylase activity was present in the enzyme preparation, free methyl methionine sulfonium was eliminated as the possible intermediate.

Fromm & Nordlie (173) purified from rat liver thetin-homocysteine transmethylase of comparable purity to that obtained from horse liver. Homoserine was found to act as a competitive inhibitor with respect to homocysteine, but no evidence was obtained for the formation of O-methylhomoserine. Shapiro & Yphantis (174) presented evidence that the microbial homocysteine transmethylases are metalloenzymes while the mammalian enzymes are not.

Mudd (175) has reported partial purification from bakers' yeast of an enzyme which cleaved S-adenosyl-L-methionine to 5′-methylthioadenosine and

α-amino-γ-butyrolactone; the reaction was not reversible. Evidence was presented (176) that the carboxyl oxygen of S-adenosyl-L-methionine displaced methylthioadenosine by direct nucleophilic attack of the γ-carbon atom adjacent to the sulfonium center. De la Haba & Cantoni (177) described an enzyme which specifically condensed adenosine and L-homocysteine to yield S-adenosyl-L-homocysteine. Chemical methylation of this compound yielded the sulfonium diastereoisomers of S-adenosyl-L-methionine (178). This product was 50 per cent effective in the enzymic methylation of guanidinoacetic acid and catechol, and in the enzymic cleavage by yeast. The inactive stereoisomer was prepared by enzymic resolution, and its configuration was established as (+)-S-adenosyl-L-methionine. Guanidinoacetate methylpherase utilized S-adenosyl-D-methionine at a much slower rate than the L-isomer.

The six "extra" methyl groups of the porphyrin-like moiety of vitamin B_{12} were shown to be derived from C^{14} methyl-labelled methionine (179). Betaine and choline were inactive in this respect.

Cystathionine.—In continuation of their studies on crystalline homoserine deaminase-cystathionase, Matsuo & Greenberg (180) were able to dissociate the enzyme into pyridoxal-5-phosphate and inactive apoenzyme. The coenzyme was required for activity with homoserine, cystathionine, and cysteine. Possible modes of binding of the coenzyme to the apoenzyme and the mechanism of inhibition of enzyme activity by certain inhibitors were discussed. By using radioactive cystathionine and unlabelled homoserine as a trap, they (181) demonstrated that there was no release of free homoserine when cystathionine was cleaved by the enzyme. On the other hand, the enzyme did catalyze the incorporation of homoserine into cystathionine in the presence of cysteine. The mechanism of action which they proposed was removal of the γ-substituent from the α-aminobutyrate moiety of the substrate, resulting in the formation of the unsaturated vinylglycine; the latter then undergoes intramolecular rearrangement and final hydrolysis to yield ammonia and α-ketobutyrate. Selim & Greenberg (182) purified from rat liver a cystathionine synthetase which catalyzed the synthesis of cystathionine from serine and homocysteine. Although the enzyme was free from cystathionase and homocysteine- and cysteine-desulfhydrases, it exhibited serine deaminase activity. Evidence was presented that one enzyme was most likely responsible for both the synthetase and the deaminase activity. Hope (183) showed that there was no correlation between the distribution of cystathionine and its synthetase in brain.

Taurine.—Stern & Stin (184) found that x-irradiation did not alter the concentration of taurine in muscle, spleen, thymus, or liver of rats, although there was a marked increase in urinary excretion of taurine. They expressed the view that dietary methionine was the major source of urinary taurine. On the other hand, Kay & Entenman (185) showed that excess taurine could not be formed through the oxidation of methionine-S^{35}, whereas excess inorganic sulfate did arise as a result of extra-hepatic methionine oxidation.

Frendo *et al.* (186) reported that blood platelets contain 1.5 mg. of taurine per gm. of platelets and that they convert cysteine to taurine.

Cavallini *et al.* (187) reported a new compound in the urine of rats fed a diet containing 6 to 12 per cent L-cysteine. The compound was identified as thiotaurine (aminoethylthiosulfonate). The natural occurrence of hypotaurine in molluscs (188) and of taurobetaine in sponges (189) have also been reported.

The presence of S-methyl-L-cysteine in kidney beans was originally reported by Thompson *et al.* (190). Zacharius *et al.* (191) have now isolated and identified γ-glutamyl-S-methyl-L-cysteine from kidney beans. This dipeptide represented a third of the nonprotein nitrogen fraction of the mature bean. Horner & Kuchinskas (192) showed that, in rats, the methyl group of S-methyl-L-cysteine was more extensively oxidized to CO_2 than that of methionine and that the methyl group derived from the former compound may be incorporated into choline and creatine. Confirmatory evidence for the participation of leucine or mevalonic acid in the formation of felinine, an S-alkyl derivative of cysteine, was obtained by Avizonis & Wriston (193) who used C^{14}-labelled compounds.

Proline and Hydroxyproline

Origin of hydroxyproline in collagen.—Further evidence that free hydroxyproline is not an intermediate in the formation of collagen hydroxyproline was presented by Green & Lowther (194). By use of slices of carrageenin-induced granuloma tissue, they were unable to demonstrate significant incorporation of L-C^{14}-hydroxyproline into collagen. Furthermore, the addition of unlabelled hydroxyproline to an incubation medium containing C^{14}-proline had no effect on the ratio of specific activities of collagen proline and hydroxyproline. Similarly, Sinex *et al.* (195), when they fed or injected labelled hydroxylysine into young rats, could not observe significant incorporation of the label into hydroxylysine of the skin collagen. Green & Lowther (194) observed, as did other investigators, that the specific activity of hydroxyproline from neutral salt-soluble collagen increased from approximately 0.6 to 1.6 times that of proline during the 4 hr. incubation. To account for this variable ratio, they postulated the existence of independent intermediary pools between free and bound proline and hydroxyproline; the hypothesis is incompatible with hydroxylation of proline after completion of the collagen peptide chain.

Robertson & Schwartz (196) had previously shown that, in granulomas of scorbutic guinea pigs, a hydroxyproline-poor protein, which displayed some collagen-like properties, accumulated. It was suggested then that ascorbic acid was required for the hydroxylation of proline bound in this supposed precursor of collagen. However, recent analysis of this protein by Robertson *et al.* (197) has revealed that it does not even contain sufficient amounts of proline and glycine to be considered a collagen precursor. Gross (198) also showed that the skin of severely scorbutic guinea pigs does not

contain any noncollagen protein rich in proline and glycine. Thus, the concept that protein-bound proline (procollagen) is hydroxylated during collagen synthesis is without support. However, the nature of the precursor for collagen hydroxyproline is yet to be elucidated.

Mitoma *et al.* (199) demonstrated a direct incorporation of administered C^{14}-hydroxyproline into collagen by chick embryos. However, even in this rapidly developing system, hydroxyproline was only one-eighth as effective as proline as a precursor for collagen-bound hydroxyproline. The parallelism between the specific activities of free and collagen-bound hydroxyproline following administration of C^{14}-proline to chick embryos indicated that these two might be derived from a common precursor, possibly an activated form of hydroxyproline. The scheme set forth in Figure 5 might explain the findings in the chick embryo.

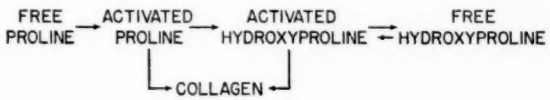

FIG. 5. Proline-hydroxyproline-collagen relationships.

It has been proposed that proline must first be activated before it can be converted to hydroxyproline. The activation of free hydroxyproline may be the limiting step in its incorporation into collagen. Katz & Goss (200) showed that formation by *Streptomyces antibioticus* of the hydroxyproline-containing actinomycin I could be increased several-fold by the addition of hydroxyproline to the growth medium. This might represent a direct incorporation of hydroxyproline into a peptide.

There have been claims that ascorbic acid is involved in the hydroxylation of proline to hydroxyproline (196, 197, 201). However, the possibility still exists that the increase in hydroxyproline which is observed following administration of ascorbic acid to scorbutic animals may represent an overall stimulation of collagen synthesis independent of the hydroxylation of proline.

Metabolism of proline and hydroxyproline.—A full description of studies on the conversion of L-hydroxyproline to L-glutamate by extracts of *Pseudomonas striata* has appeared (202). The utilization of all four isomers of hydroxyproline could be induced with either L-hydroxyproline or D-allohydroxyproline, both being characterized by the L-configuration at the hydroxylcarbon. The epimers, having the D-configuration at the same carbon, L-allo- and D-hydroxyproline, were not inducers. Evidence for α-ketoglutarate as a precursor of glutamate in the degradation of hydroxyproline was presented. Roche & Ricaud (203) reported that proline oxidase from mitochondria (prepared in an isotonic sucrose solution containing ethylenediamine tetraacetate) was active and stable. Yura & Vogel (204) purified

pyrroline-5-carboxylate reductase from *Neurospora crassa*. The possible role of this enzyme in proline synthesis was discussed.

Ketoproline.—The administration of 4-keto-L-proline to chick embryos was shown to increase free hydroxyproline levels several-fold (205). The underlying mechanisms for the observed increases in hydroxyproline were found to be inhibition of hydroxyproline oxidation and enzymic conversion of ketoproline to hydroxyproline. The occurrence of ketoproline in actinomycin formed by *Streptomyces* was reported by Brockmann & Manegold (206). Since proline, hydroxyproline, and ketoproline occur in actinomycin, the biosynthetic interrelationships of these imino acids pose an interesting problem.

Histidine

A detailed description of studies relating to the origin of the imidazole ring of histidine has appeared (207). The amide-N of glutamine was directly incorporated into the N-1 position of the imidazole ring by *E. coli*, and the N-1 and C-2 atoms of purines were incorporated as a unit into the N-3 and C-2 positions of histidine. Conversely, evidence was presented that histidine donated not only a formyl group, but also a nitrogen atom, in the formation of purine precursors (208). Thus it was found that the inhibition of growth of *E. coli* by azaserine could be reversed by histidine, urocanic acid, formamidinoglutaric acid, and glutamine.

Serveral new metabolites of histidine have been reported. Hydantoin-5-propionate was isolated and identified from the urine of rats following administration of urocanic acid (209). This metabolite was also identified in the urine of monkey and man following the intravenous injection of labelled histidine. Brown (210) reported the presence of another metabolite of histidine-C^{14} representing approximately 14 per cent of the urinary radioactivity. The compound showed marked acidic properties and did not appear to be on the histamine or urocanic acid pathways of histidine degradation. Uranaka (211 to 214) isolated four crystalline products after incubating urocanic acid with a supernatant fraction from rabbit liver; they were identified as aminoparabanic acid, aminoglutaconimide, α-ketoglutaric acid amide, and isoaminoglutaconic acid amide. It has previously been reported that an extract of *Pseudomonas aeruginosa* converted urocanic acid to hydantoin acrylic acid when ethylenediamine tetraacetate was present in the incubation medium. The same compound was produced by the rabbit liver extract in the presence of $10^{-2}M$ ethylenediamine tetraacetate. On the other hand, the bacterial extract decomposed urocanic acid to hydroxyisoglutamine in the absence of the chelating agent. A partial explanation of these diversified results may be that the ethylenediamine tetraacetate stabilized the imidazolone compound thus permitting additional oxidation to the hydantoin compound.

Feinberg & Greenberg (215) have been able to study in more detail the decomposition of urocanic acid. It had been thought that the action of urocanase on urocanic acid yielded *N*-formimino-L-glutamic acid. However,

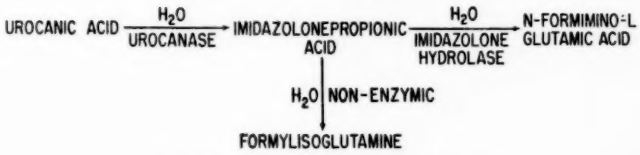

FIG. 6. Decomposition of urocanic acid.

the present report deals with a partially purified urocanase preparation which does not form formiminoglutamic acid. The initial compound formed appeared to be imidazolonepropionic acid. A non-enzymic decomposition product of imidazolonepropionic acid was also identified as formylisoglutamine. The reaction sequence is shown in Figure 6.

Winnick & Winnick (216) demonstrated that an enzyme preparation from chick muscle catalyzed the formation of carnosine and anserine from their constituent amino acids. ATP and Mg^{++} were required, and only the L form of histidine was utilized. Anserine formation proceeded more readily than carnosine formation. Anserine could also be formed from carnosine by direct methylation of the latter (217). S-adenosyl-L-methionine was active in this respect, but methionine was not. Carnosine was a better methyl acceptor than histidine, but guanidinoacetic acid was inactive under the same experimental conditions. A similar relationship was found in the transethylation reactions from S-adenosyl-ethionine. Kalyankar & Meister (218) showed that the requirement for ATP and Mg^{++} could be obviated when β-alanyl-adenylate was used in place of β-alanine. β-Alanylphosphate was inactive. Addition of CoA did not affect the rate of peptide synthesis, whereas pyrophosphatase was stimulatory. This apparently, constitutes the first unequivocal demonstration of an enzymic utilization of an amino acid adenylate in peptide bond synthesis.

The metabolic role of carnosine still remains in doubt. Jencks & Hyatt (219) have shown that the stimulation of glycolysis and oxidative phosphorylation, which has been attributed to carnosine by Severin & Meshkova (220) and by others, can be explained by the heavy metal complexing properties of this peptide. Jencks & Hyatt (219) found no evidence for enzymic or non-enzymic transfer of phosphate from carnosine phosphate to any constituents of a crude extract of rat muscle. An observation (221) that carnosine or anserine could prevent the deterioration of oxidative phosphorylation in aged mitochondria may be explained on the same basis.

Since the betaine of histidine and ergothioneine occur together in several biological systems, Ackermann *et al.* (222) have suggested that the synthetic route must be histidine→histidine-betaine→ergothioneine. That the intact histidine molecule is incorporated into ergothioneine was substantiated by Melville *et al.* (223) who used uniformly labelled N^{15}-histidine and showed that the imidazole ring and the trimethylammonium group were both highly labelled. They also showed that in a methionineless mutant of

Neurospora crassa, all three of the methyl groups were derived intact from methionine.

Fink *et al.* (224) reported that rabbits placed on vitamin-E deficient diets excreted 1-methylhistidine. Methylhistidinuria preceded creatinuria and increased progressively. Preformed anserine in the muscle was ruled out as the source of this amino acid.

Histamine and diamines.—Rothschild & Schayer (225) have shown that the histidine decarboxylase in rat peritoneal fluid arises primarily from the mast cells. It was readily shown that pyridoxal phosphate was the coenzyme, since, in this tissue, it was loosely bound to the enzyme. Inhibitors of histidine decarboxylation were also studied. Schayer *et al.* (226) have also shown an increase in histidine decarboxylase activity in rat skin following administration of 48/80 (a "histamine-releasing agent"). Histamine formation has also been demonstrated with a minced preparation of canine mastocytoma (227).

The original observation of Kobayashi (228) on the N-methylation of histamine has been extended. By use of the soluble fraction of mouse liver, Lindahl (229) found that S-adenosylmethionine is an efficient methyl donor to histamine. The N-methyl transferase was partially purified from mouse liver and found to be different from catechol-O-methyl transferase (230).

Niwa *et al.* (231) showed that rats sensitized with *Bordetella pertussis* had considerably reduced histaminase activities in lung, brain, and liver than did the controls. There were no changes in succinoxidase and monoamine oxidase activities. This observation seems to account for the histamine hypersensitivity of rats thus sensitized. Purification of histaminase has yielded preparations having the same specific activities toward other diamines as they do toward histamine (232). Similar inhibitions were observed with isoniazid, and the author concluded that, in his purified preparation, histaminase and diamine oxidase could not be separated.

Razin *et al.* (233) studied the degradation of polyamines by various bacteria. *Pseudomonas aeruginosa* oxidized spermine, spermidine, putrescine, agmatine, and cadaverine. Spermidine, propane-1,3-diamine, and γ-aminobutyric acid were identified as degradation products of spermine metabolism by this organism. In another organism, *Serratia marcescens*, propane-1,3-diamine accumulated during the breakdown of spermine. Various aspects of diamine metabolism were discussed.

Phenylalanine and Tyrosine

Phenylalanine.—Kosuge & Conn (234), in a continuation of their studies on coumarin biosynthesis in sweet clover, have shown that both phenylalanine and transcinnamic acid are precursors of coumaric acid. Their data indicate that orthocoumaric acid isomerizes and lactonizes to form coumarin. Here also, shikimic acid appears to be the precursor of aromatic compounds. Coumarin was rapidly metabolized in these preparations, and melilotic acid was shown to be one of the products formed. Other studies in higher plants

by McCalla & Neish (235, 236), using leafy cuttings of *Salvia*, have also shown that shikimic acid is a good precursor of aromatic amino acids. In this system, there was little interconversion of phenylalanine and tyrosine and there was no breakdown of these amino acids to glutamate. Cinnamic, dihydrocinnamic, (−)-phenyllactic acids, and L-phenylalanine were good precursors of phenolic acids while D-phenylalanine, L-tyrosine, and (+)-phenyllactic acid were poor in this respect. These results support a hypothesis of lignin biosynthesis involving substitution of the aromatic ring of intermediates that have the cinnamic acid side chain. The ability of tyrosine to serve as a precursor of lignin in certain grass species may be attributed to the ability of these plants to convert *p*-hydroxyphenylacetic acid to *p*-hydroxycinnamic acid (237).

Bruns (238) has shown that both L-erythro and L-threo phenylserine can be formed enzymically from benzaldehyde and glycine in sheep liver. L-Threo-β-phenylserine was isolated, and the cleavage of this compound was investigated. It was also shown that *p*-nitrophenyl-β-L-serine was formed from *p*-nitro benzaldehyde and glycine.

Camien & Dunn (239) have shown that L- and D-phenyllactic acid can replace L-phenylalanine in *Lactobacillus casei* but are not interchangeable as a D-α-hydroxy acid source in this organism. The authors concluded that there may be distinct D- and L-phenyllactic dehydrogenases.

Kaufman (240) has further investigated the mechanism of the conversion of phenylalanine to tyrosine. It is known that this reaction requires two enzymes, reduced pyridine nucleotide, and oxygen (241, 242) and a pteridine-like cofactor (243). By use of stoichiometric amounts of 2-amino-4-hydroxy-6,7-dimethyl-tetrahydropteridine, the initial rate of tyrosine formation was found to be independent of both TPNH and one of the enzymes (sheep liver enzyme). In the absence of TPNH, the conversion of phenylalanine to tyrosine was accompanied by the oxidation of the tetrahydropteridine to an intermediate which was rapidly converted to dihydropteridine. The proposed scheme is set forth in Figure 7. Kaufman & Levenberg (244) have further purified the two liver enzymes and the naturally occurring cofactor. The most active pteridine in their enzyme system was 2-amino-4-hydroxy-6-methyltetrahydropteridine. Although many functional tests on the naturally occurring cofactor gave negative results, the material did give an atypical Pauly reaction and a characteristic fluorescence

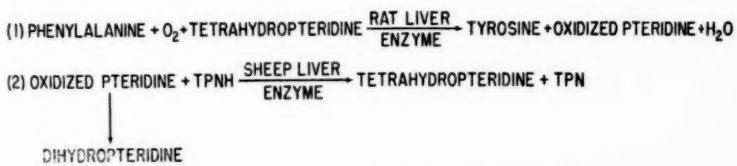

FIG. 7. Conversion of phenylalanine to tyrosine.

after alkali treatment. Folic acid antagonists (aminopterin and amethopterin) inhibited the reaction *in vitro*. *In vivo*, these compounds also raised blood phenylalanine levels, suggesting an inhibition, *in vivo*, of the conversion of phenylalanine to tyrosine, although this was not conclusively shown.

The exact manner by which phenylalanine is converted to *o*-hydroxyphenylacetic acid is still not clear, although Tashian (245) has shown that phenylpyruvic acid administration results in an increased excretion of *o*-hydroxyphenylacetic acid by man. The author therefore suggested that *o*-tyrosine may not be an intermediate in the conversion of phenylalanine to *o*-hydroxyphenylacetic acid.

Tyrosine metabolism.—Sereni *et al.* (246) have studied the factors that influence the development of tyrosine-α-ketoglutarate transaminase activity in rat liver. Livers of fetal animals had 10 to 20 per cent of the activity present in adult liver. Two hours after birth, activities increased rapidly and reached maximum values 12 hr. after birth; these were twice the usual adult levels. Adrenalectomy caused a 12-hr. delay in the development of enzyme activity after birth, but this effect could be reversed by hydrocortisone. Kenney (247) has purified the enzyme 100- to 150-fold by fractional chromatography on diethylaminoethyl cellulose columns and showed that it was inhibited by sulfhydryl reagents.

Anderson *et al.* (248) have shown that livers of rats subjected to tourniquet injury have three times more tyrosine-α-ketoglutarate transaminase activity than do normal rat livers. It was shown that the normal system contained two components, one of which was increased to a much greater extent in the traumatic state than the other. Kinetic studies showed that the rate of reaction of tyrosine and pyridoxal phosphate was increased in the traumatic state. Lin *et al.* (249), in a study of tissue concentrations of six enzymes involved in aromatic amino acid metabolism, have shown that only tyrosine-α-ketoglutarate transaminase exhibits a true sex difference in tissue concentration.

Zannoni & LaDu (250), in a continuation of their studies on tyrosine degradation via homogentisic acid, have shown that *p*-hydroxyphenylpyruvic acid oxidase is inhibited by excess substrate and that this inhibition can be prevented by both ascorbic acid and 2,6-dichlorophenolindophenol.

Segal & Mologne (251), by use of a liver sulfate transfer system, have tested the ability of various tyrosine derivatives to accept sulfate. Only those compounds that did not contain a carboxyl group or a substituted carboxyl group but possessed an unsubstituted amino group were active. Tyramine was therefore active in this system. The high pH optimum (9.3) suggested the need for an uncharged amino group. The oxidation of tyrosine and tyramine by horseradish peroxidase and H_2O_2 has been studied by Gross & Sizer (252). The hydrochloride of dityramine [2,2′-dihydroxy-5,5′-bis-(β-ethylamino)-diphenyl] was isolated following incubation with tyramine. The presence of the terphenyl derivative was also indicated. Tyrosine, in this system, underwent similar reactions.

Zioudrou & Fruton (253) have shown that both L-tyrosyladenylate and glycyl L-tyrosyladenylate are incorporated non-enzymically into mitochondrial proteins, while the C^{14}-tyrosyl residue of tyrosinamide is incorporated via an enzymic process that involves a peptidase-catalyzed transamidation reaction.

Catecholamines.—The biosynthesis of the catecholamines is known to proceed via the following pathway: tyrosine→3,4-dihydroxyphenylalanine (DOPA)→3,4-dihydroxyphenylethylamine→norepinephrine. Studies on DOPA decarboxylase have shown that phosphopyridoxal-isonicotinyl hydrazone is more active than pyridoxal phosphate in stimulating enzyme activity (254). The molecule was active in its intact form, as it was not split to the coenzyme in the process. Creveling & Udenfriend (255) have shown that the enzyme which hydroxylates 3,4-dihydroxyphenylethylamine to norepinephrine (dopamine-β-oxidase) is present in brain, where it is localized in brain stem areas. There is evidence that this side chain oxidation requires both ATP (256) and ascorbic acid (257). Chemical and enzymic studies on the mechanism of the oxidation of 3,4-dihydroxyphenylethylamine were carried out by Senoh *et al.* (258) using a mixture of 3,4-dihydroxyphenylethylamine-β-β-H^3 and-α-C^{14}. The fate of the tritium atoms on the benzyl carbon during chemical and enzymic oxidation to norepinephrine and other oxidation products was determined. It was found that in the course of the conversion only one tritium atom was lost. In his studies on the conversion of tyrosine to *p*-hydroxymandelonitrile, Gander (259) has demonstrated a similar type of reaction involving side chain hydroxylation in plants. Cell-free extracts were also obtained which converted tyrosine to *p*-hydroxyphenylserine. Drell (260) has reported that stimulated sympathetic nerves convert C^{14} tyrosine to catecholamines to a greater extent than do non-stimulated fibers.

The major route of metabolism of injected norepinephrine and epinephrine is via methylation to the corresponding 3-methoxy-4-hydroxy derivatives (261). The methoxy amines and a small percentage of the catecholamines are oxidized by monoamine oxidase to the corresponding acids. Goldstein and coworkers (262) have shown that methylation is involved in the metabolism of injected 3,4-dihydroxyphenylethylamines. Sixty per cent of the injected material was recovered as 3-methoxy-4-hydroxy phenylacetic acid, while only 2 to 3 per cent was recovered as 3-methoxytyramine. When a monoamine oxidase inhibitor was employed, 31 per cent of the 3,4-dihydroxyphenylethylamine could be recovered as 3-methoxytyramine and 20 per cent of the original hydroxytyramine was excreted unchanged in the urine. Thus, with this amine, oxidative deamination as well as 0-methylation appear to proceed. In brain homogenates (263), the bulk of the metabolism was via initial oxidative deamination to 3,4-dihydroxyphenylacetic acid.

Senoh *et al.* (258) demonstrated the presence of a new metabolite of 3,4-dihydroxyphenylethylamine, 2,4,5-trihydroxyphenylethylamine, in rat urine

following injection of the labelled precursor. The compound was formed readily during nonenzymic oxidation of the dihydroxy amine. A method of separating this metabolite from norepinephrine by selective 0-methylation was also described.

Bacq (264), Udenfriend *et al.* (265), and Axelrod & Laroche (266) have shown that pyrogallol and other catechol compounds readily inhibit the methylation of catecholamines both *in vivo* and *in vitro*. The catechols appear to act as competitive substrates of 0-methylpherase. It was also shown that glycocyamine and other non-catechol methyl acceptors could appreciably inhibit 0-methylation *in vivo* (265). An excellent summary of the various aspects of the biochemistry and physiology of the catecholamines has recently been published (267).

Monder & Waisman (268) have shown that the copper-catalyzed oxidation of DOPA to melanin pigments was inhibited by plasma from leukemia and cancer patients more than by normal plasma.

Dennell (269), in a continuation of his studies on the developing insect cuticle, has investigated the roles played by catechol, protocatechouic acid, DOPA, and hydroquinone in this process. It appears the hydroquinone, but not catechol, is responsible for hardening of the puparium.

Thyroxine.—Yamazaki & Slingerland (270) have shown that the deiodination, *in vitro*, of thyroxine derivatives is inhibited by nitrogen, heavy metals, cyanide, and versene. Radioactive studies with the perfused liver (271) showed that thyroxine and triiodothyronine were concentrated in the liver and that small amounts of I^{131} could be found in the bile. Twenty-six per cent of the triiodothyronine was deiodinated in 12 hr.. while 8 per cent of the thyroxine was deiodinated in the same period of time. Intact rat kidney mitochondria were found to deiodinate thyroxine and triiodothyronine (272) and also to convert both thyroxine and triiodothyronine to the corresponding acetic acid derivatives (272, 273). Sonically disrupted mitochondria (272), however, lost their deiodination activity but retained the ability to form the acetic acid analogues; this was true for several tissues. The ability of rats, both to deiodinate and convert triiodothyronine to its acetic acid analogue, has also been studied by Roche *et al.* (274). Nakano *et al.* (275) studied the metabolism of mono- and diiodotyrosine, triiodothyronine, and thyroxine with a cobra venom preparation. The corresponding α-keto and acetic acids were formed, plus inorganic iodide from mono- and diiodotyrosine. It appeared that deiodination occurred after deamination. The possibility of a non-enzymic deiodination of the iodothyronine derivatives has been suggested by Muzard & Triantaphyllidis (276).

Potter *et al.* (277) have shown that the mammary gland of the lactating rat is a major pathway for the elimination of I^{131}. Sixty-one per cent of the I^{131} excreted in this way in 6 hr. was protein-bound. After 24 hr., 86 per cent of the I^{131} excreted in this way was protein-bound.

The sulfate conjugate of triiodothyronine was found in bile following administration of thyroxine (278). The acetic acid metabolite of triiodothyronine has also been shown to be converted to its corresponding sulfate

derivative and to appear in plasma (279). The intestinal flora were found to play only a minor role in the metabolism of the sulfate derivative of triiodothyronine (280).

A non-enzymic model reaction (281) for the conversion of 3,5-diiodotyrosine desamino analogues to the corresponding analogues of thyroxine was studied at pH 7.5 at 37° C. The propionic acid derivative was found to be the best substrate for this reaction.

Trytophan

Synthesis.—Reed *et al.* (282) have studied the mechanism whereby *p*-aminobenzoic acid inhibits *Saccharomyces cerevisiae.* The inhibition was reversed by the aromatic amino acids, including tryptophan, but not by shikimic acid. In the presence of *p*-aminobenzoic acid, shikimic acid levels were increased, indicating that the inhibition occurred at a stage between shikimic acid and the aromatic amino acids.

Srinivasan (283) demonstrated that a cell-free extract of an *E. coli* mutant readily synthesized anthranilic acid from shikimic acid-5-phosphate and L-glutamine. Shikimic acid, itself, plus L-glutamine was inactive, although the addition of ATP yielded weak activity. L-Aspartic acid, L-glutamic acid, and L-asparagine could not replace L-glutamine in this system; DPN or TPN was also required.

Yanofsky (284) has shown that the tryptophan synthetase of *E. coli* also participates in the conversion of indoleglycerol phosphate to indole. Certain mutants that were unable to convert indole to tryptophan synthesized a protein (CRM) immunologically related to tryptophan synthetase, which converted indoleglycerol phosphate to indole. Similarly, in tryptophan-requiring mutants of *Neurospora crassa*, Suskind & Jordan (285) have shown that the CRM protein, antigenically related to the wild type tryptophan synthetase, possesses indoleglycerol phosphate-synthesizing activity. Some alteration of the protein may have eliminated the activity of the protein with respect to serine but did not affect the ability to combine with indole. Doy & Gibson (286) have shown that mutant strains of *Aerobacter aerogenes* and *E. coli* that require tryptophan or indole for growth, accumulate a new compound, 1-(*o*-carboxyphenylamino)-1-deoxyribulose, which may be the immediate precursor of indolylglycerol phosphate.

Nicotinic acid pathway.—Tanaka & Knox (287) have purified tryptophan pyrollase (tryptophan oxidase-peroxidase) from *Pseudomonas* and have shown that it is similar to the liver enzyme, an iron porphyrin protein, active in the ferrous state. The role of peroxide in this reaction has now been elucidated. The mechanism appears to involve a direct oxygenation of the substrate with molecular oxygen by the ferrous enzyme. Peroxide is then utilized to reconvert the ferric form of the protein into the active ferrous form. The authors reiterate that the more common name used for this enzyme, tryptophan oxidase peroxidase, is now a misnomer and that the name, tryptophan pyrrolase, first suggested by Kotake & Masayama, (288), is correct.

Several new findings concerning the adaptive nature of the animal

enzyme have also appeared. Civen & Knox (289) showed that hydrocortisone induction of tryptophan pyrrolase in rat liver was not associated with increased levels of free tryptophan in the liver of normal animals or with a significant increase of tryptophan metabolites by pyridoxine-deficient animals. Induction by tryptophan, on the other hand, increased both free tryptophan in liver and the excretion of its metabolites. The inducing effect of hydrocortisone and tryptophan when administered together, was found to be the sum of their separate actions. Preincubation of liver slices with tryptophan increased the tryptophan pyrrolase activity by almost 50 per cent, suggesting that induction could occur *in vitro*. No such activity was observed with hydrocortisone. Induction by cortisone, *in vivo*, was found to be inhibited by ethionine (290), and the inhibition was reversed by methionine. Hypothalamic lesions or hypophysectomy were shown to decrease the response of tryptophan pyrrolase to histidine (291). The enzyme was not detectable in rat liver until 12 days of age (292) but appeared before this time when tryptophan was administered. The activity of the enzyme increased twofold from 38 to 400 days of age and was also increased by bovine growth hormone (293). Senescent animals showed no greater difference in their adaptive response than did mature animals (294). Dubnoff & Dimick (295), in noting the stabilizing effect of substrate on the lability of the enzyme, pointed out that this could be a possible explanation of the tryptophan effect, i.e., not synthesis of new protein but decreased destruction of existing enzyme. Although the evidence for the latter is by no means conclusive, it does point up the controversial aspects of this whole field. Although in their studies Civen & Knox (289) found no induction in homogenates, Clouet & Gordon (296) reported an increase in tryptophan pyrrolase activity in cell-free homogenates from livers of young rats when tryptophan was added to a medium containing an amino acid mixture and ATP. However, Gordon & Rydziel (297) in a second report showed that the apparent induction was a reflection of mitochondrial integrity and did not involve *de novo* synthesis of enzyme protein.

Experiments in the rat have shown that radioactivity from DL-tryptophan-α-C^{14} is incorporated into other amino acids such as serine, alanine, aspartate, and glutamate (298). The labelling pattern suggested that pyruvate-2-C^{14} was the intermediate and was consistent with the kynurenine-hydroxyanthranilic acid pathway. Other C^{14} studies by this same group (299) offered conclusive evidence that 3-hydroxyanthranilic acid is an intermediate in the oxidative metabolism of tryptophan.

Stevens & Henderson (300) have solubilized the recently discovered kynurenine hydroxylase from liver mitochondria. K^+ ion doubled the activity of dialyzed preparations. Although riboflavin-deficient animals had only 30 to 50 per cent of the activity found in control animals, no definite requirement for flavin coenzymes could be shown *in vitro*. The same workers (301) have investigated some of the requirements for 3-hydroxyanthranilic acid oxidase. Dialyzed aged preparations from beef liver required both Fe^{++} and

reduced glutathione for maximal activity and sulfhydryl groups appeared to be necessary for activity.

Tracer studies by Moline *et al.* (302) on the conversion of 3-hydroxyanthranilic acid to nicotinic acid have shown that C_3 of hydroxyanthranilic acid becomes the α-carboxyl of quinolinic acid and is recovered as CO_2 in the conversion to nicotinic acid. Hydroxyanthranilic acid oxidase has been shown to be inhibited by vitamin K as well as by 2-acetylaminofluorene and N-2-fluorenyldiacetamide (303, 304). The latter two compounds are known to produce bladder tumors in animals. *In vivo*, tryptophan and 3-hydroxyanthranilic acid reversed the inhibition of vitamin K. In experiments, *in vitro*, DPN was inactive with respect to vitamin K, but with the two latter inhibitors it was effective in reversing the inhibition.

Rat kidney kynurenine transaminase was shown to be competitively inhibited by a variety of mono- and di-carboxylic acids (305). Mason postulated that there are two cationic groups on the enzyme which are involved in binding the inhibitors and substrate.

More quinoline derivatives have been identified as normal tryptophan metabolites. Vitamin-B_6 deficient rats were shown to excrete 8-hydroxyquinaldic acid (306). Thus far, seven quinoline derivatives have been identified in normal urine; quinaldic acid, 8-hydroxyquinaldic acid, quinaldylglycine, kynurenic acid, xanthurenic acid, 6-hydroxykynurenic acid, and 8-methyl ether of xanthurenic acid. The various quinoline derivatives could be separated from other tryptophan metabolites by adsorption on Dowex 50 (H^+) and elution with water (307). Sylianco & Berg (308) have studied the effect of riboflavin deficiency on the accumulation of tryptophan metabolites by kidney and liver slices. The vitamin deficiency lowered the indolepyruvic acid accumulation in the medium, but other metabolites, such as xanthurenic acid, kynurenine, kynurenic acid, and anthranilic acid, were all increased.

Further studies have helped to elucidate the oxidative pathway of quinoline degradation in *Pseudomonas*. Behrman & Tanaka (309) showed that cell-free extracts degraded kynurenic acid with the utilization of three moles of O_2 and the liberation of three moles of CO_2. Trytophan labelled in the β position yielded no $C^{14}O_2$. Further studies on this pathway by Hayaishi *et al.* (310) showed that glutamate and acetate were formed from kynurenic acid. These latter authors indicated that the carboxyl carbon and C_2, C_3, C_4 and C_{10} of kynurenic acid become the carbon atoms of glutamate.

In the rat, a major metabolite of nicotinic acid has been shown to be N-methyl-4-pyridone-5-carboxylamide (311), along with N-methylnicotinamide, N-methyl-2-pyridone-5-carboxyamide, and nicotinamide. Nicotinamide N-oxide is also formed from nicotinamide in the rat (312) and can be readily reduced by liver homogenates to nicotinamide. Another pathway of nicotinic acid metabolism in *Pseudomonas* involves hydroxylation to 6-hydroxynicotinic acid. Hunt *et al.* (313) have studied the distribution of

the enzyme in this organism and found the bulk of it to be present in the cell wall and protoplast membrane. A smaller percentage was found in some of the lighter particles.

Butenandt *et al.* (314) have continued their studies of the ommochrome pigments. Sulfur was shown to be present, and both xanthurenic acid and 2-amino-3-hydroxyacetophenone were released under alkaline conditions of hydrolysis. 3-Hydroxykynurenine was formed by acid hydrolysis. Joshi & Brown (315) have described an animal system which converts 3-hydroxykynurenine to a pigment similar to that described by Butenandt; mitochondria and cytochrome-*c* were needed for pigment formation.

Supportive evidence from the work of Henderson *et al.* (316) has again indicated that, in corn and tobacco, tryptophan is not the precursor of niacin: tryptophan-7-α-C^{14} and 3-hydroxyanthranilic acid were not incorporated into the vitamin by these plants. Studies on C^{14}-tryptophan in the chick embryo (317) have confirmed earlier reports that this amino acid is the precursor of niacin.

Rothstein & Greenberg (318) have presented evidence that suggests a new oxidative pathway of tryptophan metabolism. Tissue extracts in 0.9 per cent KCl converted 7-C^{14}-tryptophan to $C^{14}O_2$, while kynurenine labelled in the equivalent position on the benzene ring was metabolized to a much smaller extent. These results were not obtained if sucrose was used in the preparation of the tissues.

Indoleacetic acid pathway.—There is now good evidence that indoleacetic acid, a normal constituent of urine, is formed from tryptophan in the tissues. Two possible routes by which this conversion may take place are shown in Figure 8.

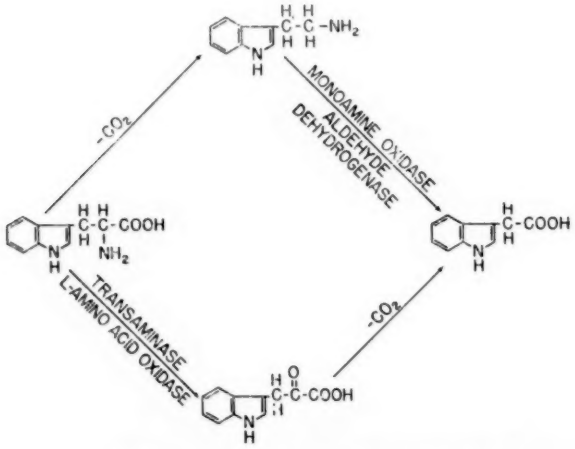

FIG. 8. Pathways leading to indoleacetic acid in animals.

Weissbach *et al.* (319), by means of a simple chemical method for indoleacetic acid assay in urine and tissues, showed that the normal excretion in man is 5 to 18 mg./day. Most of the acid formed by mammalian tissues and by fecal bacteria arose via transamination. Evidently some came from tryptamine, since liver, kidney, and human fecal bacteria were able to decarboxylate tryptophan to yield tryptamine. This amine is an excellent substrate of monoamine oxidase and, *in vivo*, would readily be converted to indoleacetic acid.

Sjoerdsma *et al.* (320) have shown that between 40 to 100 μg. of tryptamine per day are normally excreted by man; this excretion was markedly elevated in patients treated with monoamine oxidase inhibitors. The conclusive evidence that tryptamine is a normal metabolite of tryptophan initiated studies on the metabolism of this amine. Although tryptamine is most readily oxidized by monoamine oxidase, Jepson *et al.* (321) have shown that a microsomal preparation can hydroxylate this amine in the presence of TPNH to 6-hydroxytryptamine. Previous reports (322) of microsomal hydroxylation of tryptamine to 5- and 7-hydroxytryptamine could not be confirmed. The significance of this pathway, *in vivo*, remains in question. Similar studies by Szara & Axelrod (323) have been done with N,N-dimethyltryptamine; this compound was also enzymically demethylated.

Lin and coworkers (324, 325) have shown that there are two independent tryptophan transaminases in rat liver and that these differ from the transaminases which act on other aromatic amino acids. One of the enzymes required α-ketoglutarate as the amino acceptor, the other, pyruvate. The former was adaptive, and its level could be increased by hydrocortisone, and also by substrate in the presence of adrenal hormones (326). Lin *et al.* (324) have demonstrated the presence of an enzyme that catalyzes the enol-keto tautomerization of indolepyruvic acid. Spencer & Knox (327), studying this enzyme in more detail, have shown that glutathione functions as a coenzyme.

5-Hydroxyindole pathway.—The conversion of tryptophan to 5-hydroxyindole compounds has received much interest over the past few years. The major pathway in animal tissues involves the steps set forth in Figure 9. No report has appeared showing the conversion, *in vitro*, of tryptophan to 5-hydroxytryptophan in mammalian tissues. However, previous studies have clearly demonstrated that tryptophan is the precursor of 5-hydroxyindole compounds (328 to 330), and a net synthesis of 5-hydroxyindole compounds has been shown to occur in *Chromobacterium violaceum* (331). A mouse mast-cell tumor which had been shown to contain large quantities of

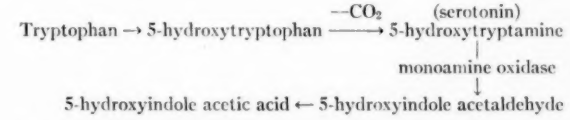

FIG. 9. Conversion of tryptophan to hydroxyindole derivatives.

serotonin (332) has been studied by Schindler *et al.* (333), and evidence was presented that these cells can synthesize 5-hydroxytryptamine. 5-Hydroxytryptophan decarboxylase has also been studied in rodent mast cells by Lagunoff & Benditt (334).

The decarboxylase from kidney has been shown to be inhibited by a variety of compounds (335) that also inhibit DOPA decarboxylase. These inhibitor studies have suggested that the same enzyme may be responsible for the decarboxylation of both DOPA and 5-hydroxytryptophan. This is in contradiction to a previous report of Clark *et al.* (336). It is apparent that more work is needed to clarify this point. Buzard & Nytch (337), in a continuation of their studies on 5-hydroxytryptophan decarboxylase, have shown that norepinephrine inhibits this enzyme in a non-competitive manner through removal of the coenzyme, pyridoxal phosphate. The non-enzymic reaction of norepinephrine and pyridoxal phosphate yielded a product which was characterized as a substituted tetrahydroisoquinoline. It has been reported that acid metabolites of phenylalanine inhibit 5-hydroxytryptophan decarboxylase when present in high concentration (338).

The metabolism of serotonin proceeds primarily through oxidative deamination catalyzed by the enzyme monoamine oxidase. The physiological roles of this enzyme have been summarized in a review by Davison (339). A soluble bacterial monoamine oxidase that oxidizes both serotonin and tyramine has also been reported (340). Since it is soluble, it may lend itself to purification, which will permit a more detailed study of its properties. Barsky *et al.* (341) have presented a further study on the mode of action of iproniazid and other hydrazides, known inhibitors of monoamine oxidase. Zile & Lardy (342) have shown that tissues of animals treated with thyroxine have lower monoamine oxidase activity. The hormonal effect was not caused by direct inhibition of the enzyme, and there was no effect on the intracellular distribution of the enzyme. Apparently, thyroxine influences the synthesis of the enzyme. A study of the amine oxidases present in plasma and a summary of the characteristics of the various known amine oxidases has been presented by Blaschko *et al.* (343).

Other minor pathways of serotonin metabolism have been shown to exist. The O-glucuronide of serotonin is formed by mice (344), and McIsaac & Page (345) have reported that several species also form N-acetyl serotonin. The latter workers have estimated that, in the animals studied, 50 to 70 per cent of the serotonin administered was metabolized via monoamine oxidase, 5 to 25 per cent was converted to N-acetyl serotonin, and 5 to 10 per cent to the glucuronide. With the monoamine oxidase inhibitors now available, it will be of interest to see what effect these compounds will have on the metabolism of serotonin by these alternate pathways.

Mansour (346) has reported an interesting effect of serotonin on carbohydrate metabolism in the liver fluke. Lactic acid production was increased two- to tenfold with no effect on volatile fatty acid formation. Epinephrine was inactive in this system.

Transamination

In many instances, transaminase reactions are intimately involved in the metabolism of an amino acid. For convenience and continuity, many of these (tyrosine, γ-aminobutyric acid, tryptophan, kynurenine, etc.) have already been discussed in the sections dealing with the appropriate amino acids.

Barban & Schulze (347) have shown that cell-free extracts of HeLa cells and mouse fibroblasts grown in tissue culture form aspartate, glutamate, alanine, glycine, phenylalanine, and tyrosine by transamination of the keto acids with a variety of amino acids. Glutamate was 9 to 16 times more effective than glutamine as an amino donor. Pyridoxal phosphate did not stimulate, and the system was not inhibited by isoniazid (isonicotinyl hydrazide).

Jenkins *et al.* (348, 349) have purified to a high degree glutamic-oxaloacetic transaminase from pig heart by use of a hydroxyapatite column. Their enzyme preparation was 80 to 85 per cent pure and contained two residues of pyridoxal phosphate. The protein acted as an indicator, being visibly yellow below pH 7.0 but colorless at pH 8.0 With various dicarboxylic acids the protonated enzyme formed complexes which inhibited its catalytic action but protected the enzyme against heat denaturation. The pyridoxal phosphate form of the enzyme was more stable than the pyridoxamine form. This same group (349) studied the kinetics of the glutamic-oxaloacetic transaminase reaction as a function of pH. Different forms of the enzyme could be identified spectroscopically at the various pH values. At low values, a complex of α-ketoglutarate with the pyridoxine group of the enzyme was present and resulted in an absorbency at 435 mμ. There was no relationship between enzyme activity and pH that could be correlated with the pK of the prosthetic group. Banks *et al.* (350) were able to separate the apoenzyme from the coenzyme and to show that the equilibrium in the reaction of the coenzyme with the apoenzyme is ten times greater with pyridoxal phosphate than with pyridoxamine phosphate.

An interesting observation on the effect of isoniazid on B_6 enzymes has been observed in two laboratories. Gonnard *et al.* (351) and Bonavita & Scardi (352) have shown that this hydrazide does not inhibit glutamate-oxaloacetate transaminase, but actually combines with pyridoxal phosphate to form the corresponding hydrazone, which stimulates the reaction. A similar effect of isoniazid was observed with DOPA decarboxylase (254). In these experiments, the hydrazone could also serve as a coenzyme. On the other hand, the cyanohydrin derivative of pyridoxal phosphate, could not serve as a coenzyme in the glutamate-oxaloacetate transaminase reaction (351). The ability to block the carbonyl group with isoniazid, with no loss in coenzyme activity, questions the role of this functional group in the enzymic reaction. However, this effect of isoniazid, although interesting, must still be accepted with some reserve, since Jenkins *et al.* (353), in studies with the highly purified transaminase did not observe it. They showed that the interaction of isoniazid with the pyridoxal form of the enzyme was accom-

panied by spectral changes that could be correlated with enzyme inhibition. The enzyme-isoniazid dissociation constant was dependent on pH. In phosphate buffer they observed two types of inhibitions, one rapid and readily reversible, the other slow and irreversible. Neither type of inhibition could be attributed to resolution of the holoenzyme.

Injections of hydrocortisone, cortisone, and prednisone into rats produced a six to thirteenfold increase in glutamic-pyruvic transaminase in one week (354, 355). Glutamate-pyruvate transaminase levels were also found to increase in animals made diabetic with alloxan. This effect was prevented by administration of insulin. Glutamate-oxaloacetate transaminase was little affected by these measures.

Miscellaneous observations.—The distribution of α-aminobutyric acid-α-ketoglutarate transaminase in animal tissues was studied by Iyer & Sukumaran (356). A phenylalanine-α-keto-β-methylvalerate transaminase has been purified twentyfold from *Neurospora crassa* (357). With this purified preparation, 13 of 19 keto and amino acids served as substrates. Transamination reactions have been studied in *Leptospira biflexa* by Markovetz & Larson (358).

Amino Acid Metabolism in Disease

Disturbances in amino acid metabolism appear in a variety of disease states. In many cases these changes can lead to procedures for early diagnosis of the disorders, while in other cases they may lead to an understanding of the disease process. Conversely, a disease state may shed light on the normal metabolism of an amino acid by exaggerating or limiting specific metabolic processes. Phenylketonuria, malignant carcinoid, alcaptonuria, and other such disorders may, therefore, be looked upon as experiments of nature. Many of the above aspects of amino acid metabolism in disease will be covered in this section.

Serum transaminase levels in disease.—A most interesting application of amino acid biochemistry to medical diagnosis has been the assay of serum transaminases, both glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase. In human organs, the former enzyme is found in highest concentration in heart: damage to heart tissue appears to result in markedly elevated levels of this enzyme in serum (359). Since this initial observation, there have been many reports concerning the use of serum glutamate-oxaloacetate and -pyruvate transaminase as a diagnostic aid in various clinical disorders. An excellent summary and review of this field was recently presented by Agress (360). In a summary of findings that have accumulated over the last five years from many laboratories, he pointed out that the correlation of serum glutamate-oxaloacetate transaminase activity with autopsy evidence of myocardial infarction was over 98 per cent. However, in the presence of cardiac failure, less than 3 per cent of the cases had elevated transaminase levels. Since this enzyme is also present in other tissues, (skeletal muscle > brain > liver > kidney > testes > lung > spleen) its

concentration in serum may also be elevated because of damage to these tissues as well. Recent studies on serum transaminase levels in a variety of clinical conditions are discussed below.

Serum transaminase levels in myocardial infarction.—An excellent correlation of electrocardiogram and clinical findings was found with glutamate-oxaloacetate transaminase in infarction and other coronary heart disease, but little correlation with glutamate-pyruvate transaminase (361). The former enzyme was superior to lactic acid dehydrogenase as a diagnostic aid in patients (362). In dogs and patients, glutamate-oxaloacetate transaminase was a most sensitive index of infarction. Glutamate-pyruvate transaminase was least sensitive in man (363). The former has been studied as a diagnostic aid in patients (364, 365). A comparison of the three enzymes in serum and in human muscle after myocardial infarction indicated a great similarity, supporting the belief that serum enzymes are escape products from necrotic tissue (366).

Serum transaminase levels in jaundice.—There was no evidence of secretion of glutamate-oxaloacetate transaminase into the bile in jaundice in conditions where the serum enzyme was elevated (367). In studies on 100 jaundiced patients, this serum enzyme and iron were found to be more accurate than any other test for liver function (368). Marked increases in serum glutamate-oxaloacetate and -pyruvate transaminases were observed in dogs following severe liver damage (369).

Toxemia of pregnancy.—Increased levels of glutamate-oxaloacetate and -pyruvate transaminases were observed in one-third of the cases examined. It was always associated with accompanying liver disorders (370).

Tumors.—Glutamate-oxaloacetate transaminase and lactic acid dehydrogenase of spinal fluid were appreciably elevated in patients with brain tumors. However, the increases could not be explained by the enzyme levels in the tumors (371). The transaminase content of some malignant tissues has been reported (372).

Stress.—An increase in serum glutamate-oxaloacetate transaminase has been observed in monkeys because of non-specific stress (373). This serum enzyme increased in humans subjected to positive radial acceleration (374).

There was no significant increase in glutamate-oxaloacetate or -pyruvate transaminase in patients with either active or inactive pulmonary tuberculosis (375), although lung does contain these enzymes. An interesting application of serum transaminase has been made by Melby *et al.* (376). Using serum glutamate-oxaloacetate transaminase as an indicator of cellular injury, they have shown that, in dogs, *E. coli* endotoxin produces cellular injury that is proportional to the administered dose, the injury increasing with time. Cortisone, when administered in large amounts, significantly reduced the leakage of glutamate-oxaloacetate transaminase into serum.

The concentrations of the free amino acids in blood, tissues, and urine are determined by overall absorption, metabolism, and excretion. Disturbances in amino acid metabolism are readily detected by changes in the amino

acid patterns in the tissues and urine. Dietary factors that contribute to the plasma levels of most of the amino acids in normal individuals have been excellently discussed by Frame (377). Additional reports on the urinary excretion of individual free amino acids by normal men and women have been presented by Soupart (378) and Leverton *et al.* (379). Christensen *et al.* (380) have indicated that with increasing age tissues lose their capacity to take up amino acids from the blood. However, it remains to be seen whether these studies which were carried out with an abnormal amino acid, α-aminoisobutyric acid, apply to all the physiological amino acids. The quantitative requirements for some of the essential amino acids have been reported to be greater for men over 50 when the total amount of nitrogen fed is increased (381). There may be some connection between these findings and those of Christensen *et al.* (380).

Hsien *et al.* (382 to 384) have shown that the N^{15} excretion, as determined by isotope concentration—time curves for ammonium ion, hippuric acid, and urea—was different in the first few hours after oral and intravenous administration of N^{15} labelled glycine, phenylalanine, and aspartate. However, after 6 hr., the patterns became essentially the same except for hippurate excretion. Problems involved in the interpretation of data on urinary nitrogen excretion were discussed with respect to the relationships of various body pools.

The effects of malnutrition, including the disease Kwashiorkor, on the free amino acids of the blood have been studied by Westall *et al.* (385). In agreement with others, they reported a general aminoacidopenia. Their findings also suggested that the amino acid patterns reflect marked interference with the development and activity of enzymes concerned with the metabolism of amino acids. Disturbances in deamination and in metabolism of cystine were indicated. Of particular interest was the suggestion that malnutrition leads to a deficiency of the enzyme phenylalanine hydroxylase. A diminished conversion of phenylalanine to tyrosine in malnutrition has been suggested previously by others (386, 387).[4]

Many pathologic disturbances lead to abnormal urinary amino acid patterns. Mellinkoff *et al.* (388) have reported that porphyria produces marked increases in the excretion of a large number of amino acids, thus suggesting a more basic biochemical defect than abnormal porphyrin metabolism.

Beta-aminoisobutyric acid was first detected in human urine by Crumpler *et al.* (389) and Fink and co-workers (390). The latter group also demonstrated that this amino acid is derived from thymine and suggested that its excretion in the urine reflected alterations in deoxyribonucleic acid metabolism. Subsequently, Awapara (391) reported that beta-aminoisobutyric acid excretion was indeed elevated following administration of nitrogen

[4] The latter (387) is a recent translation, from the Russian, of a general review entitled "Disorders of Amino Acid Metabolism in Protein Deficiencies and Their Corrections," by S. Ya. Kaplanskiĭ.

mustards or after whole body irradiation. Rubini *et al.* (392) have recently reported marked increases in urinary excretion of this amino acid in five individuals who had been heavily exposed to mixed neutron and gamma radiation. They suggested that beta-aminoisobutyric acid excretion may be useful in the early detection of serious exposure. Gartler (393) has shown that in man thymine is probably the only significant source of urinary beta-aminoisobutyric acid and that the general population can be divided into low excretors and high excretors.

The presence in urine of an unusual substance with an odor like that of maple sugar, in certain infants with progressive familial infantile cerebral dysfunction, was first reported in 1954 by Menkes *et al.* (394). Studies by Mackenzie & Woolf (395) and by Dancis *et al.* (396) have now revealed that the odor is associated with the presence of large amounts of keto acids derived from leucine, isoleucine, and valine. Markedly elevated levels of these three amino acids were also found in urine and plasma. The situation is similar to that found in phenylpyruvic oligophrenia (see below) where the phenylalanine and phenylpyruvic acid of serum and urine are increased in concentration. These findings suggest a disturbance in the metabolism of the branched chain amino acids. The metabolism of these amino acids is known to proceed as shown in Figure 10.

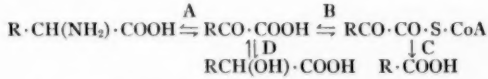

FIG. 10. Metabolism of branched chain amino acids.

Mackenzie & Woolf (395) have found large amounts of the hydroxy acids in the urine in this disease, and Dancis (397) has reported that the decarboxylation products, isobutyric, and isovaleric acids are absent. It would appear, therefore, that a metabolic block is located at stage B or C. Speculations concerning the relationship of this metabolic defect to mental function, genetic considerations of the disease, and available methods for its diagnosis were discussed further by Dancis (397) and also in an excellent editorial in the *British Medical Journal* (398).

Phenylketonuria.—Of all the disorders of amino acid metabolism, phenylketonuria has been most studied. It is now generally accepted that this disease is carried as a recessive genetic characteristic where the enzymic conversion of phenylalanine to tyrosine is markedly diminished. From our knowledge of phenylalanine metabolism, we now know that two protein catalysts, reduced pyridine nucleotides and an unknown cofactor, are required to convert phenylalanine to tyrosine.

In earlier studies (399, 400), it was shown that enzyme 1 (see Figure 7, p. 231) is present in adequate quantities in livers of phenylketonuric individuals but that enzyme 2 was markedly diminished. In a recent paper by Kaufman (401) these findings have been corroborated. In addition, he has shown that

the hydroxylation cofactor is present in normal amounts in phenylketonuric iver. Thus, the only direct biochemical lesion that can be demonstrated in this mental disorder is one involving a liver enzyme; all other metabolic changes appear to be secondary to the block in phenylalanine metabolism. Thus, the apparent block in melanin formation which has been reviewed by Dancis (397), results from an excess of unmetabolized phenylalanine which inhibits tyrosinase activity. The changes in indole excretory products and the lowered excretion of the serotonin metabolite, 5-hydroxyindoleacetic acid, in this disorder are also secondary to the block in phenylalanine hydroxylation, since these all return to normal on low-phenylalanine diets. Pare *et al.* (402) and Baldridge *et al.* (403) have suggested that the diminished excretion of 5-hydroxyindoleacetic acid is caused by an inhibition of serotonin formation. The former have suggested that the effect is attributable to inhibition of 5-hydroxytryptophan decarboxylation, while the latter relate it to inhibition of tryptophan hydroxylation. Although either of these is possible, a third, and more likely, explanation of the somewhat lower urinary excretions of 5-hydroxyindoleacetic acid may be interference with renal function. The changes in blood serotonin, reported by the former group (402), may conceivably be attributed to effects of phenylalanine or its products on platelet function.

Another interesting urinary metabolite which is found in large amounts in phenylketonurics is *o*-hydroxyphenylacetic acid. This also arises from phenylalanine and falls to normal values when patients are placed on a phenylalanine-free diet. Tashian (245) has now reported that phenylpyruvic acid can be converted to *o*-hydroxyphenylacetic acid in normal individuals and may therefore be the parent substance of the hydroxy acid in phenylketonurics.

One of the most exciting aspects of this disorder is that there may be some hope of preventing the onset of mental defects by administration of low-phenylalanine diets. Under such conditions normal phenylalanine blood levels are observed and phenylpyruvic acid is no longer excreted. Previous reports concerning effects of such diets in older infants and children have been variable. However, Horner & Streamer (404) have recently reported that three infants treated from earliest infancy with such a diet have attained ages of $3\frac{1}{2}$, $2\frac{1}{4}$, and 1 yr. and have normal motor and mental patterns. In each of the cases there have been untreated phenylketonuric siblings who showed marked impairment. Since there have been reports of untreated phenylketonurics with near-normal function [one such was recently reported by Leonard & McGuire (405)], one cannot be certain that the healthy pattern in the three cases reported by Horner & Streamer (404) has resulted from the dietary treatment. However, such a pattern of development in three successive phenylketonurics is suggestive, and it will be worth following the development of these and other such cases.

Catecholamines.—Catecholamine metabolism was investigated in patients with pheochromocytoma by Sjoerdsma *et al.* (406), and enzymes involved

in the biosynthesis of norepinephrine were shown to be present in the tumor. 3-Methoxy-norepinephrine was also formed by homogenates of the tumor and was found to be excreted in the urine in large quantities. Tumor norepinephrine was found to have a relatively short half life (*ca.* 10 hr.) as compared to values (several days) previously found for catecholamines in animal adrenal glands by Udenfriend & Wyngaarden (407). It was suggested that tumors containing only norepinephrine are of neural origin and that the rapid synthesis and secretion is representative of sympathetic nerves; whereas tumors which contain epinephrine as well are of adrenal origin, and, in these, synthesis is a slow process although storage capacity is great. The latter is comparable to the situation in the adrenal medulla.

Another instance of tyrosine metabolism associated with a tumor concerns malignant melanoma. There have been many papers written on this disease. However, the whole subject has been brought up to date in an excellent review by St. Geme (408). In this article are discussed the most recent concepts of melanin formation and melanocyte proliferation as well as the clinical and therapeutic aspects of the disorder.

Hydroxyproline and connective tissue.—Hydroxyproline and hydroxylysine are unque in that, in animals, they are found almost entirely in the collagen of connective tissue. Although one would expect alterations in the metabolism of these amino acids in connective tissue disorders, Ziff *et al.* (409) were unable to demonstrate any unusual urinary excretion of hydroxyproline in patients with a variety of the so-called collagenous diseases. More recently, Sjoerdsma *et al.* (410) were able to demonstrate what appears to be a specific increase in hydroxyproline-containing peptides in the urine of patients with Marfan's Syndrome. A case report and review of the clinical manifestations of this disorder has recently been published (411). In further studies on hydroxyproline metabolism in man, Prockop *et al.* (412) have found that the urinary excretion of hydroxyproline peptides was not related to the diet but that it reflected the metabolic breakdown of body collagen. The high excretion of hydroxyproline in patients with Marfan's Syndrome suggested either an abnormally large pool of collagen or an increased rate of turnover.

There exists a defect of dietary origin called lathyrism, which results from the ingestion of the seeds of the sweet pea, *Lathyrus odoratus*. The condition is caused by the presence of beta-aminopropionitrile and can be brought on experimentally by administering the latter compound. Experimental lathyrism has been reviewed recently by Gardner (413). Since it has been compared to Marfan's Syndrome, it may provide a tool with which to study alterations of collagen and hydroxyproline metabolism. In experimental lathyrism caused by the administration of beta-aminopropionitrile or aminoacetonitrile, it has been generally agreed that there is no obvious chemical alteration of connective tissue in the afflicted animals (414 to 417). However, marked physical alterations could be demonstrated, indicating that these agents affected the states of aggregation of the collagen fibrils.

Pedrini (418) reported that the enzymic synthesis of hexosamine from glutamine and glucose-6-phosphate in the epiphyseal plates of rabbits with lathyrism was decreased by 76 per cent.

5-Hydroxyindole metabolism.—Much information has been obtained on 5-hydroxyindole metabolism in man by studies on patients with malignant carcinoid (419 to 423). These early studies showed that the tumor produces large amounts of serotonin, and it would appear that many of the symptoms that make up the total syndrome are attributable to the increased serotonin levels in the body. Since the major route of metabolism of serotonin in animals and in man is via oxidative deamination, large quantities of 5-hydroxyindoleacetic acid are excreted in the urine of these patients. Based on the greatly increased excretion of this acid by carcinoid patients, a simple colorimetric test was developed for diagnosis (424). However, certain precautions must be employed in evaluating this test. The patient should not be on medication, since it has been shown that acetanilide gives a weak, but false, positive reaction caused by a metabolic product, *p*-hydroxyacetanilide, while chloropromazine or its metabolites inhibits the urinary color reaction. The large amounts of serotonin and catecholamines in bananas (425) could also lead to false positive carcinoid tests as well as the false positive urinary catechol tests used in the diagnosis of pheochromocytoma (426).

The following procedure should be employed when carcinoid is suspected. The patient should be on a known diet on the day the urine is collected and should be receiving little, if any, medication. The direct color assay (424) should be done on two urine samples, one containing an added, known amount of 5-hydroxyindoleacetic acid to determine whether color formation is being inhibited. If a positive test is observed, the urine should be reassayed, using the specific extraction procedure of Udenfriend *et al.* (427). In almost all instances where the latter test has been positive and where typical carcinoid symptoms existed, the presence of a carcinoid tumor has been subsequently confirmed. A vast survey of clinical conditions has failed to yield a false positive result (428). However, McMullen & Hanson (429), employing the direct color reaction, have obtained positive reactions in two patients with no evidence of carcinoid tumor, even upon surgical exploration. Since no details were presented, it is difficult to determine whether these findings represent some hitherto unknown disturbance in serotonin metabolism or some interference with the color assay.

In the past year, several reports describing studies on carcinoid patients have appeared (430 to 432), and Schneckloth *et al.* (433) have studied the metabolism of serotonin in one such patient. As usual, the excretion of 5-hydroxyindoleacetic acid was high, and small amounts of serotonin were present. In addition, serotonin-O-glucuronide and some unidentified metabolites were present. However, examination of the tumor indicated that serotonin was not the major 5-hydroxyindole present; another 5-hydroxyindole compound was detected in ten times the concentration of serotonin.

Following observations that phenylacetic acid could inhibit decarboxy-

lation of 5-hydroxytryptophan, *in vitro* (338), Sandler & Close (434) administered this acid to an atypical patient with malignant carcinoid who excreted both serotonin and 5-hydroxyindoleacetic acid. They observed some decrease in the urinary excretion of the acid and serotonin, which was consistent with inhibition of 5-hydroxytryptophan decarboxylase. In a second paper (435), administration of 5 gm. of phenylacetic acid to five typical carcinoid patients produced what appeared to be a significant lowering of the urinary 5-hydroxyindoleacetic acid in three of them. No significant alleviation of the symptoms of carcinoid was observed. On the basis of these experiments, Sandler and his colleagues suggested that phenylacetic acid inhibits 5-hydroxytryptophan decarboxylase *in vivo*. A more likely explanation of the effects of phenylacetic acid on 5-hydroxyindoleacetic acid excretion was suggested by Milne (436). He pointed out the marked disparity between the concentrations needed to produce appreciable inhibition *in vitro* (10^{-1} to $10^{-2}M$) and the calculated tissue concentrations of a 5 gm. dose to a patient (10^{-3} to $10^{-4}M$). He also pointed out that excretion and metabolism of phenylacetic acid are very rapid. Milne suggested that phenylacetic acid, like other aromatic acids, is excreted by proximal tubular secretion. Such acids exhibit a mutually competitive inhibition and one would expect 5-hydroxyindoleacetic acid excretion to be diminished for this reason alone. His conclusion is that in experiments, *in vivo*, it is important to distinguish changes in metabolism from changes in tissue distribution and excretion.

Attempts to show consistent altered excretions of 5-hydroxyindoleacetic acid in various pathologic states, such as schizophrenia (437), achlorhydria (438), neurocirculatory asthenia (439), and hepatic cirrhosis (440), have been unsuccessful. Although an earlier report had indicated that schizophrenic patients converted less tryptophan on a loading dose to 5-hydroxyindoleacetic acid (441), a recent study by Kopin (437) has failed to confirm this. Reserpine, which is known to release amines from their body depots, has been shown to result in an elevated 5-hydroxyindoleacetic acid excretion in man during the first 24 hr. (442), but not thereafter. Donaldson *et al.* (440, 443) have reported that 5-hydroxytryptophan is converted more efficiently to 5-hydroxyindoleacetic acid in patients with hepatic cirrhosis than in normals. The conversion of serotonin to 5-hydroxyindoleacetic acid was not affected.

Borges *et al.* (444) have shown that patients with hepatic coma show altered electroencephalograms following low doses of 5-hydroxytryptophan; it is evident that the relationship of liver disease to 5-hydroxytryptophan metabolism should be investigated further. The serotonin content of platelets from patients with inflammatory or rheumatoid diseases was reported to be slightly lower than in normals (445). Administered extravascular serotonin was reported to cause exaggerated responses in patients with rheumatoid arthritis (446). These were diminished if a serotonin antagonist was also administered.

Price *et al.* (447) have studied the excretion of tryptophan metabolites arising from the kynurenine-nicotinic acid pathway in a variety of neurological and psychiatric diseases. A significant alteration was seen in porphyric patients: increased excretions of kynurenine, acetyl-kynurenine, kynurenic acid, and hydroxykynurenine. Although tryptophan derivatives have been implicated in cancer of the bladder, no major alteration in the excretion of tryptophan metabolites was found in the urine of Turkish cows with urinary bladder cancer (448).

Hartnup's syndrome (449) is characterized by a general aminoaciduria with increased excretion of indican, indole acetic acid, and indolylacetylglutamine. Milne *et al.* (450), using tryptophan-loading experiments, have shown that the indole acids were excreted in large amounts by these patients for as long as 24 hr. after a test load of the amino acid; in normals, the peak indole acid excretion occurred in the first 2 hr. It also appeared that less kynurenine was formed in these patients from a given dose of tryptophan.

An abnormal hydroxyskatole, present as the sulfate conjugate, was found in the urine of patients with sprue (451, 452). Injection of skatole to rats yielded the same hydroxyskatole derivative, and it could also be prepared chemically from skatole by use of the iron ascorbic acid hydroxylating system (453). The material has now been identified as 6-hydroxyskatole (454).

In studies employing *Mycobacterium tuberculosis*, tryptophan (and histidine to a lesser degree) has been found to be an excellent tuberculostatic agent. It also showed activity, *in vivo*, when tested in infected guinea pigs (455).

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LIPID METABOLISM[1,2]

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Since a broad review of the many facets of lipid biochemistry is impossible in the assigned space, a detailed analysis of a rather small area of the field has been undertaken. Thus, the chemistry and analytical methodology of lipids and the biochemistry of phospholipids will not be reviewed here. For other aspects of the field not reviewed here, the reader should consult the review by Law (1) in this volume on the complex lipids, as well as the reviews on steroids by Popják (2), terpenes by Bloch (3), lipid chemistry by Klenk & Debuch (4), and lipid metabolism related to higher plants by Stumpf & Bradbeer (5) and to physiological problems by Popják (5a). This year, a new periodical entitled *Journal of Lipid Research* (6) joins the rapidly increasing family of journals covering restricted fields.

Fatty Acid Oxidation

Enzymes of the β-oxidation cycle.—Research has continued on the details of the β-oxidation enzyme systems. Steyn-Parvé & Beinert (7) have observed that the three acyl dehydrogenases of pig heart form unusually firm complexes with their substrates. The enzyme-substrate complex will stand prolonged dialysis, ammonium sulfate precipitation, and treatment with Dowex 2 and charcoal. The substrate is bound as an acyl-CoA-protein complex rather than as an acyl-protein complex. No randomization with either external CoA or acyl moieties is observed. When the green butyryl dehydrogenase of pig liver was first purified (8), copper appeared to be an essential component of the enzyme system. Steyn-Parvé & Beinert (9) have re-examined this enzyme and report that copper can be readily removed from the protein by dialysis against versene. The spectrum and activity are not significantly changed by this treatment. The green butyryl dehydrogenase can be transferred into a yellow form by a variety of reagents, including reductants, CoA, etc. This yellow form without the green pigment appears to be more active. Copper loss is not associated with the change of the green to the yellow form of the enzyme.

Crystalline crotonase, which catalyzes the hydration step of the fatty

[1] The survey of the literature pertaining to this review was concluded in October, 1959.

[2] The following abbreviations are used: AMP for adenosine monophosphate; ATP for adenosine triphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); P_i for orthophosphate; PP for pyrophosphate; ThPP for thiamine pyrophosphate; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form).

acid cycle, had already been shown to be not only a hydrase but also a positional isomerase and possibly a *cis→trans* isomerase (10). Stern (11) now reports that large amounts of crystalline crotonase catalyze two additional reactions: (*a*) transfer of the crotonyl moiety of crotonyl-S-pantetheine to CoA to form crotonyl CoA (transcrotonylase), and (*b*) the racemization of D(−) β-hydroxybutyryl CoA to the L(+) isomer (racemase). Throughout the purification procedure involving successive crystallization and aging of the enzyme, the ratio of crotonase to transcrotonylase activities was constant. The turnover number of transcrotonylase at pH 7.5 and 25° is 450. Stern (12) has presented evidence for two racemases in ox liver extracts, one of which is related to crotonase (9 per cent of total activity) and the remainder to a specific β-hydroxybutyryl CoA racemase. The turnover number of the racemase, associated with crotonase, is 27 at pH 9.5 and 25° and may not play too important a role in fat metabolism. The multiple functions of crotonase are of considerable interest. On the basis of the formation of a transitory carbonium ion on the β-carbon, hydration, *cis→trans* isomerization, and racemization can be explained. The reaction, transcrotonylation, can be interpreted as a displacement of the thioester group by a nucleophilic attack by a SH compound, such as CoA, on the thioester carbon having a fractional positive charge. However, the positional isomerization catalyzed by crotonase is difficult to explain, particularly because of a requirement of activation on the carbon atoms β and γ to the thioester carbon.

Stern (12) has presented further evidence for a specific β-hydroxybutyryl racemase, which differs from the coupling mechanism of D(−) β-hydroxybutyryl dehydrogenase and L(+) β-hydroxybutyryl dehydrogenase proposed by Wakil (13).

L(+) β-Hydroxybutyryl dehydrogenase has been isolated from pig heart as a crystalline protein (14). It has a turnover number of 42,000 per 100,000 gm. enzyme at pH 7.0 at 25°. The optimum pH for reduction of acetoacetyl CoA was 6 to 7 while the oxidation of L(+) β-hydroxybutyryl CoA was 9.6. At the respective pH optima, the reduction was five times faster than the oxidation. At pH 7, these relative rates were 18 to 1. At pH 9.6, the apparent equilibrium constant K' is

$$K' = \frac{(\text{acetoacetyl CoA})\ (\text{DPNH})\ (H^+)}{[\text{L}(+)\ \beta\text{-hydroxybutyryl CoA}]\ (\text{DPN}^+)} = 2.7 \times 10^{-10}. \qquad 1.$$

The crystalline protein appears to have a small amount of DPN^+ bound to it.

The preparation and biological properties of the acyl adenylates have been studied by Vignais & Zabin (15), Whitehouse, Mocksi & Gurin (16), and Srere (17). Wainfan & van Bruggen (18) discuss some of the hazards involved in the reaction of hydroxylamine with thioesters, and they tabulate the R_f values of some 28 hydroxyamates determined in two solvent systems.

Deacylases.—A rather large number of deacylases have now been described in literature. They include acetyl CoA deacylase (19), succinyl CoA deacylase (19, 20), β-hydroxyisobutyryl CoA and β-hydroxypropionyl

deacylase (21), β-hydroxymethylglutaryl CoA deacylase (22), and palmityl deacylase (17, 23).

Acetoacetyl CoA deacylase has also been reported in several tissues (24) but Bublitz (25) and Lynen *et al.* (26) have recently presented evidence for a rather unique cycle. The so-called acetoacetyl deacylase of liver was examined by Bublitz [(25); Lynen *et al.* (26)] who separated the activity into two separate protein fractions. When these purified fractions were recombined with synthetic acetoacetyl CoA, no free acetoacetate was formed. The missing factor was found to be acetyl CoA. The two proteins were identified as the β-hydroxy-β-methyl glutaryl CoA condensing enzyme described by Rudney (27, 28) and the β-hydroxy-β-methyl glutaryl CoA cleavage enzyme (29). The reaction sequence is depicted as follows:

$$\text{Acetyl CoA} + \text{acetoacetyl CoA} \underset{\longleftarrow}{\xrightarrow{\text{condensing enzyme}}} \beta\text{-hydroxy-}\beta\text{-methyl glutaryl CoA} + \text{CoA} \qquad 2.$$

$$\beta\text{-Hydroxy-}\beta\text{-methyl glutaryl CoA} + H_2O \xrightarrow[\text{enzyme}]{\text{cleavage}} \text{acetoacetate} + \text{acetyl CoA} \qquad 3.$$

$$\text{Net reaction: acetoacetyl CoA} + H_2O \rightarrow \text{acetoacetate} + \text{CoA} \qquad 4.$$

The carboxyl carbon of free acetoacetate is derived exclusively from the carboxyl carbon of acetyl CoA [(26); see also discussion on page 274]. Lynen uses the term "β-hydroxyl-β-methyl glutaryl CoA cycle" to describe reactions 2, 3, and 4 and has invoked it to explain the inequality of labeling observed between the carboxyl carbons of acetoacetic acid. Stern & Miller (192), however, report that, in rat liver or in ox liver mitochondria, the condensing and cleavage enzymes can be completely blocked by iodoacetamide, but acetoacetyl CoA is still specifically cleaved to acetoacetate by a specific deacylase.

Ketone substances.—McCann (30) compared oxidations of L(+) β-hydroxybutyrate, D(−) β-hydroxybutyrate, and acetoacetate by washed mitochondria from rat liver, kidney, heart, and brain. With the exception of liver mitochondria, the mitochondria of all the other tissues could oxidize acetoacetate. Brain mitochondria oxidize only D-β-hydroxybutyrate appreciably, but mitochondria from liver, kidney, and heart oxidize both isomers. It was concluded that, in these tissues utilizing D-β-hydroxybutyrate, the acid is oxidized by D(−) β-hydroxybutyric dehydrogenase to acetoacetic acid. The L(+) isomer is first converted to its CoA derivative, oxidized to acetoacetyl CoA, and then cleaved by thiolase to acetyl CoA and metabolized via the Krebs cycle. The liver is unique in that it can not further utilize free acetoacetate, which explains in part the propensity of this organ to excrete ketone substances into the blood. The interrelationship can be shown in Figure 1.

Role of β-hydroxybutyrate in bacterial photosynthesis assimilation.—In 1927, Lemoigne (31) discovered highly refractile granules in cells of *Bacillus megaterium* and identified them as polyesters of β-hydroxybutyric acid.

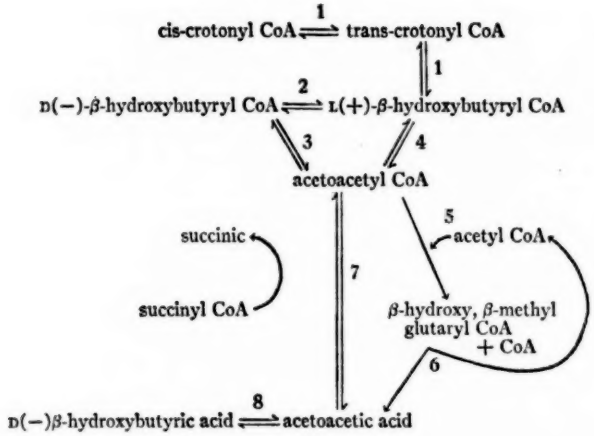


FIG. 1. Interrelationship of acetoacetic acid and its precursors: 1, crotonase; 2, racemase; 3, D(−)-β-hydroxybutyryl dehydrogenase of Wakil; 4, L(+)-β-hydroxybutyryl dehydrogenase; 5, condensing enzyme; 6, cleavage enzyme; 7, succinyl CoA transuccinylase; 8, D(−)-β-hydroxybutyric acid dehydrogenase.

Forsythe (32) has found the polyesters to be widespread in a large number of non-photosynthetic, aerobic, Gram-negative bacteria. Doudoroff *et al.* (33) observed that *Pseudomonas saccharophila* stored 60 to 90 per cent of assimilated carbon derived from glucose, acetate, or butyrate as the polyester. When the external substrate is removed, the ester is rapidly broken down as a source of endogeneous carbon. Stanier *et al.* (34) report that, in the photometabolism of acetate and butyrate by resting cells of *Rhodospirillum rubrum*, the acids are converted largely to poly-β-hydroxybutyrate, and the reductive synthesis is coupled either with an oxidation of part of the acid or with an uptake of molecular hydrogen. The enzyme systems which are involved in the synthesis of the polyester in *R. rubrum* have not been investigated, but it is reasonable to assume that, if acetate is assimilated, the following reactions take place:

$$\text{ADP} + \text{P}_i \xrightarrow{\text{light}} \text{ATP} \qquad 5.$$

$$\text{Acetate} + \text{ATP} + \text{CoA} \longrightarrow \text{acetyl CoA} + \text{AMP} + \text{PP} \qquad 6.$$

$$2\ \text{acetyl CoA} \longrightarrow \text{acetoacetyl CoA} + \text{CoA} \qquad 7.$$

$$\text{Acetoacetyl CoA} + \text{H}_2 \longrightarrow \beta\text{-hydroxybutyryl CoA} \qquad 8.$$

$$n(\beta\text{-Hydroxybutyryl CoA}) \longrightarrow \text{poly-}\beta\text{-hydroxybutyric} + n(\text{CoA}) \qquad 9.$$

Stanier *et al.* (34) conclude that, when the rate of uptake of exogeneous substrate is greater than the rate of gross cellular synthesis, the two principal reserve materials in purple bacteria are at least poly-β-hydroxybutyrate and a polysaccharide. Substrates that are readily converted to acetyl units

without the intermediate formation of pyruvate yield mostly the polyester; on the other hand, substrates that yield pyruvic acid with a concomitant generation of reducing power form primarily polysaccharide. Polyesters are of considerable value to the cell since (a) their precursors are acidic—conversion to the polyester yields a neutralized, osmotically inert compound; (*b*) the ester serves as an intracellular reserve of reducing power; and (*c*) an endogeneous supply of carbon can be accumulated when it is not needed for cell synthesis and is readily made available when the exogeneous supply fails.

Oxidation by bacteria and molds.—A systematic survey of the β-oxidative enzymes was made with cell-free preparations of H 37 Ra strains of *Mycobacterium tuberculosis* (35). β-Hydroxy acyl dehydrogenase, enoyl hydrase, and β-keto acyl thiolase activity were observed, the enzymes were partially purified, and the usual kinetics were investigated. The properties appear to be very similar to those found in animal tissues. The functioning state of these enzymes would indicate the presence of the β-oxidative cycle in this bacterium.

Webley *et al.* (36), using washed cells of *Nocardia opaca*, studied the β-oxidation of substituted butyric acid. Thus γ-phenoxybutyric acid is oxidized more slowly than γ-phenylbutyric. Substitution in the ortho position of the phenoxy derivative further retards the reaction. β-Hydroxy acids can be isolated as intermediates.

A vibrio (37) grown on C_6 to C_{10} fatty acids yielded extracts that oxidized these acids but not acetate, propionate, butyrate, or valerate. The odd-numbered fatty acids (heptanoic and nonanoic acids) were oxidized to acetate and propionate. The cofactors ATP, CoA, DPN, and Mg^{++} were essential for activity.

Franke *et al.* (38) prepared extracts of *Phycomycetes* and *Ascomycetes* that degraded long chain fatty acids to the methyl ketones. A fatty acid dehydrase and the stable β-keto carboxylase were detected.

It has been known that the mold *Penicillium roqueforti* metabolizes fatty acids to methyl ketones with one less carbon atom than its fatty acid precursor. Gehrig & Knight (39) observed that at some stage in mold growth ketone formation declines. They present evidence that the site of ketone formation is in the spores rather than in the mycelial bodies. If the spores are permitted to germinate, ketone formation ceases.

Oxidation of hydrocarbons.—Biological oxidation of saturated hydrocarbons is an intriguing puzzle. By using enrichment culture techniques, organisms can be obtained that can readily metabolize chemically inert paraffins. Thus, Harris (40) has examined the oxidation of saturated hydrocarbons by a micrococcus strain isolated from the soil-asphalt interface of Kansas highways. Tentatively identified as *Micrococcus paraffinae* Sohngen, the organism was grown on a medium with hydrocarbons as the sole source of carbon. Washed suspensions vigorously oxidized a homologous series of hydrocarbons. The acid homologues were oxidized two to three times more

rapidly than the hydrocarbons, but the alcohols were relatively inert. Interestingly, decane is relatively inert as a substrate in contrast to octane, dodecane, or tetradecane. Steric studies with Hirshfelder molecular models revealed that only decane of the 8-, 12-, 14- series could assume a compact ring-like structure without exposure of a terminal methyl group. Presumably, the enzyme protein responsible for the primary oxidation will only attack exposed methyl groups. Since the organism can be readily grown and has high oxidative capacity for these hydrocarbons, they should provide an excellent source for enzyme mechanism investigations.

Another approach is that of Leadbetter & Foster (41), who have studied the obligate methane-utilizing bacterium *Pseudomonas methanica*, which grows rapidly at the expense of methane gas as the only carbon source. The following reactions have been reported:

$$\text{Ethane} \xrightarrow{O_2} \text{acetic} + \text{acetaldehyde} \qquad 10.$$

$$\text{Propane} \xrightarrow{O_2} \text{propionic} + \text{acetone} \qquad 11.$$

$$\text{Butane} \xrightarrow{O_2} \text{butyric} + \text{2-butanone} \qquad 12.$$

Curiously, while the organism can metabolize the homologues of methane, it can only utilize methane for growth. The earlier work of Senez (42), Webley (43), and Leadbetter (44) should also be consulted.

Hepatic oxidation of fatty acids.—When rats are fasted for one day and maintained at a low temperature (0–2°), their liver becomes heavily infiltrated with fat with a concomitant decrease in liver glycogen (45). Liver slices obtained from these animals show a serious defect in the formation of $C^{14}O_2$ from acetate-1-C^{14} and a number of short chain fatty acids. Addition of glucose to the slices restores the oxidative ability to a normal rate. However, long chain fatty acids are oxidized at the normal rate, although addition of glucose will cause a marked stimulation. In sharp contrast, liver slices from rats fed and maintained at 25°C. have a lower capacity to oxidize long chain fatty acids when glucose is added, demonstrating the sparing action of glucose in long chain fatty acid oxidation. Masoro & Felts (45) have concluded: (*a*) that some phase of carbohydrate metabolism was essential for maximal rate of hepatic fatty acid oxidation, and (*b*) no relation to the "sparking" action of Krebs cycle intermediates existed, since these could not substitute for glucose. In recent studies on this problem, these workers (46) prepared liver homogenates from fed rats maintained at 25°C. and from fasted rats maintained at 0–2° for 24 hr. Addition of ATP to the liver homogenates from cold-fasted rats resulted in a marked stimulation of $C^{14}O_2$ from acetate-1-C^{14}. Furthermore, P/O studies showed that almost complete uncoupling of oxidative phosphorylation occurs in liver of cold-fasted rats. They concluded that the following disturbance occurs in the hepatic metabolism of cold-fasted rats: (*a*) an uncoupling of oxidative phosphorylation, and (*b*) a high

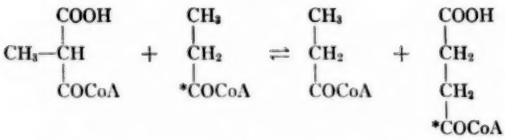
rate of gluconeogenesis, which competes with fatty acids for the small amount of available ATP. Further implications of their findings of the heat production mechanism in animals exposed to cold have been reported (47).

α-Oxidation in higher plants.—In 1956, Stumpf (48) reported that a long chain fatty acid peroxidase catalyzes the decarboxylation of long chain fatty acids in the presence of H_2O_2. A variety of H_2O_2-generating systems were effective in supplying this component to the peroxidase system. A direct correlation could be shown between formation of long chain aldehydes and the decarboxylation of carboxyl-labeled long chain fatty acids. Imidazole and azide strongly inhibited the reaction. Martin & Stumpf, in 1959 (49), showed that two enzymes are involved in the internal oxidation of long chain fatty acids. The first is a fatty acid peroxidase, which peroxidatively decarboxylates the acid to an aldehyde and CO_2. The aldehyde is then oxidized by a DPN^+ specific dehydrogenase to an acid containing one less carbon than the initial acid substrate. The α-oxidative system is probably restricted to the C_{18} to C_{14} acids, the C_{15} acid being the most active substrate. With those acids below C_{14}, the peroxidase is not able to decarboxylate.

Propionic Acid Metabolism

Several pathways have now been elucidated for the degradation of propionic acid. In animal tissues (50 to 52), propionate is oxidized by a sequence that involved: (*a*) conversion into propionyl CoA; (*b*) an ATP-dependent carboxylation to methyl malonyl CoA; and (*c*) a quantitative conversion of the methyl malonyl CoA to succinate, which then enters the Krebs cycle for further transformation.

The isomerization step (*c*) has been further examined by Beck & Ochoa (53). Since methyl malonyl CoA has an asymmetric carbon atom, only one-half of the chemically synthesized compound but all of the enzymically synthesized methyl malonyl CoA is isomerized to succinyl CoA. The enzymatic carboxylation of propionyl CoA yields the enantiomorph of methyl malonyl CoA, which serves as the substrate for the isomerase. When enzymic isomerization of methyl malonyl CoA→succinyl CoA is carried out in the presence of C^{14}-labeled propionyl CoA, C^{14}-labeled succinyl CoA is formed. The specific activities of propionyl CoA and succinyl CoA are essentially the same. No incorporation of C^{14} is observed in the presence of unlabeled propionyl CoA and $C^{14}O_2$ when these compounds are substituted for C^{14}-labeled propionyl CoA. The authors concluded that the isomerization occurs through a transcarboxylation wherein the carboxyl groups of methyl malonyl CoA (or succinyl CoA) are transformed to an acceptor molecule of propionyl CoA, which is thereby converted either to succinyl CoA or methyl malonyl CoA with the simultaneous release of one molecule of propionyl CoA (Reaction 13). This is an unusual reaction, particularly since it is difficult to envisage a fractional negative charge on the β-carbon of propionyl CoA, which is the required site for CO_2 addition in order to yield



13.

succinyl CoA. However, vitamin B_{12} may be implicated in this reaction. Marston, Allen and Smith (54) have observed that liver homogenates obtained from vitamin B_{12}-deficient sheep have considerably less capacity to convert propionyl CoA to succinate. Recently, Smith (55) has extended these observations by examining liver homogenates of vitamin B_{12}-deficient rats for the presence of propionyl CoA carboxylase and methyl malonyl CoA isomerase. He concluded that, in rats deficient in vitamin B_{12}, the activity of methyl malonyl CoA isomerse is seriously depressed, while the carboxylase remains unaffected. The relationship of this system to that described by Barker (56), wherein an enzyme isolated from *Clostridium tetanomorphum* catalyzes the isomerization of glutamate and β-methyl aspartic acid, is of considerable interest. Barker's isomerase requires as a cofactor a derivative of pseudo-vitamin B_{12} (56).

Work has continued on the elucidation of two more enzymes associated with the Flavin-Ochoa system, mainly propionyl CoA carboxylase and fluorokinase. Earlier work showed that, in crude preparations, orthophosphate is released from ATP if both CO_2 and propionyl CoA are present (51):

$$\text{ATP} + CO_2 + \text{propionyl CoA} \rightarrow \text{ADP} + P_i + \text{methyl malonyl CoA} \qquad 14.$$

In the absence of propionyl CoA but in the presence of fluoride, a CO_2-dependent reaction between ATP and fluoride yielded ADP and monofluorophosphate. This was called the fluorokinase reaction (51). In order to clarify the mechanism of the carboxylation step, both fluorokinase and carboxylase were highly purified. Tietz & Ochoa (57) crystallized fluorokinase from rabbit muscle and have found the enzyme to contain considerable pyruvic kinase activity. The following observations suggest that fluorokinase and pyruvic kinase are similar if not identical: (*a*) pyruvic kinase and flurokinase activity remain constant throughout precipitation, purification, and repeated crystallization; (*b*) similar ratios of activities are obtained when the enzyme protein is purified by different methods following either pyruvic and fluorokinase activities; (*c*) both activities require K^+ and have the same broad nucleotide specificity; (*d*) phosphoenolpyruvate and pyruvate inhibit fluorophosphate formation, (*e*) electrophoresis of the crystalline protein shows no separation of fluorokinase and pyruvic kinase activities (57, 58). In the meantime, Kupiecki & Coon (59) re-examined their ATP-CO_2-activating enzyme system and failed to confirm the earlier finding of Bachhawat & Coon (60). However, they have found that the ratio of the activating enzyme to pyruvic kinase activity is fairly constant. Moreover, crystalline pyruvic kinase can substitute for their activating enzyme in their assay system. Thus, a zinc- CO_2^+-, and NH_2OH-dependent cleavage of ATP and

a magnesium ion-, CO_2^+-, and fluoride-dependent cleavate of ATP appear to have in common all the properties of pyruvic kinase.

$$\left\{\begin{array}{l} \text{ATP} + (CO_2) + NH_2OH \xrightarrow{Zn^{++}} \text{ADP} + (CO_2) + (\text{P—NHOH})? \\ \text{ATP} + (CO_2) + F^- \xrightarrow[K^+]{Mg^{++}} \text{ADP} + (CO_2) + F^-PO_3H_2 \end{array}\right\} \quad 15.$$

$$\text{ATP} + \text{pyruvic} \xrightarrow[Mg^{++}]{K^+} \text{ADP} + \text{phosphoenol pyruvate}$$

Tietz & Ochoa (61) have purified the propionyl CoA carboxylase some 500 times from pig heart extracts. This extensive purification failed to resolve the system into separate enzymes for CO_2 activation and propionyl CoA carboxylase. The purified enzyme has, however, no fluorokinase activity, and crystalline fluorokinase, when added to the carboxylase, had no effect on the carboxylation of propionyl CoA. The reaction is reversible.

$$\text{ATP} + CO_2 + \text{propionyl CoA} \overset{Mg^{++}}{\rightleftharpoons} \text{ADP} + P_i + \text{methyl malonyl CoA.} \quad 16.$$

Although Lardy & Adler (62) reported, in 1956, that mitochondrial extracts of biotin-deficient rat livers carboxylate propionate at a greatly reduced level, no significient amount of biotin could be detected in highly purified preparations of propionyl carboxylase (61).

Although the methyl malonate pathway appears to be the dominant one in animal tissues, Coon and his co-workers (63) have shown the following reactions to occur in the same tissues:

$$\text{Acrylyl CoA} + H_2O \rightleftharpoons \beta\text{-hydroxypropionyl CoA} \quad 17.$$

$$\beta\text{-hydroxypropionyl CoA} + H_2O \rightarrow \beta\text{-hydroxypropionic acid} + \text{CoA} \quad 18.$$

$$\beta\text{-Hydroxypropionic acid} + DPN^+ \rightleftharpoons \text{malonic semialdehyde} + DPNH + H^+ \quad 19.$$

Reaction 17 is catalyzed by crystalline crotonase and Reaction 18 by a rather specific deacylase. Reaction 19 involves β-hydroxypropionic acid dehydrogenase, which has been purified about 200 times from pig kidney extracts and is highly substrate specific since it does not attack a variety of free α- and β-hydroxy acids nor their CoA esters (64). TPN^+ is ineffective. The apparent equilibrium constant, including the H^+ as a reactant, is 9×10^{-12} in the favor of the reduced product. The enzyme is surprisingly widespread in bacteria and animal tissues.

The well-known glycogenic properties of propinate (65) and the isotopic equilibration of carbon atoms 2 and 3 in the conversion of propionate to glycogen are well satisfied by the Flavin-Ochoa system. However, malonic semialdehyde may be oxidatively decarboxylated to acetyl CoA and CO_2 or oxidized to malonyl CoA, which could then enter the fatty acid synthesis system.

Along this line, Feller & Feist (66) have observed that, in rat adipose tissue, propionate is oxidized to CO_2 at a rate equal to that in liver. However,

propionate-2-C^{14} is converted into long chain fatty acids in adipose tissue 100- to 200-fold greater than in liver slices. C^{14}-propionate is also converted into adipose fatty acids threefold greater than is methyl malonate-C^{14}.

Propionic acid metabolism in higher plants.—In higher plants, propionic acid is metabolized by a modified β-oxidative pathway to acetyl CoA and CO_2. In 1956, Stumpf & Barber (67) observed that peanut mitochondria, when supplemented with Mn^{++}, a Krebs cycle acid, ATP, and CoA, rapidly released $C^{14}O_2$ from either propionate-1-C^{14} or valerate-1-C^{14}. Giovanelli & Stumpf (68) re-examined this result and showed that propionic acid was converted to β-hydroxypropionic acid as an intermediate. It was in turn employed as the initial substrate for further studies. It could be shown that: (*a*) propionate-1-C^{14} (or β-hydroxypropionate-1-C^{14}) released all of its C^{14} as $C^{14}O_2$, and other reaction products were completely lacking in C^{14}; and (*b*) propionate-2-C^{14} (or β-hydroxypropionate-2-C^{14}) and propionate-3-C^{14} yielded $C^{14}O^2$ only after the Krebs cycle acids became labeled, but the release of $C^{14}O_2$ from carbon 3 was more rapid than from carbon 2. Acetyl CoA was neither isolated nor trapped, but succinate-C^{14} was obtained after the oxidation of either propionate-2-C^{14} or propionate-3-C^{14}. With propionate-2-C^{14}, the C^{14} was located exclusively in the methylene groups of succinate, whereas, with propionate-3-C^{14}, the C^{14} was confined to the carboxyl groups of succinate. The combined evidence suggested that: (*a*) the carboxy group of propionate is released exclusively as CO_2; (*b*) the methylene group becomes the CH_3 group of acetate; and (*c*) the methyl group of propionate is converted into the carboxyl group of acetate. The enzyme complex has also been found in mitochondria obtained from lupine cotyledons and avocado mesocarp. The sequence can be depicted by the following steps:

$$\text{Propionic acid} \rightarrow \text{propionyl CoA} \rightarrow \text{acrylyl CoA} \rightarrow \beta\text{-hydroxypropionyl CoA} \rightarrow$$
$$\beta\text{-hydroxypropionic acid} \rightarrow \text{malonic semialdehyde} \xrightarrow{\text{CoA}} \text{acetyl CoA} + CO_2 \rightarrow$$
$$\text{tricarboxylic acid cycle complex} \rightarrow CO_2 \qquad 20.$$

Propionic acid metabolism in bacteria.—In the first of a series of papers on propionic acid metabolism, Vagelos *et al.* (69) describe the purification and properties of acrylyl CoA aminase, which catalyzes the reaction:

$$CH_2 = CHCOCoA + NH_3 \rightleftharpoons CH_2NH_2CH_2CO\ CoA \qquad 21.$$

The enzyme has only been found in *Clostridium propionicum*. A wide variety of other organisms and animal tissue extracts failed to show any activity. The enzyme is inducible since extracts of *Cl. propionicum* grown on β-alanine contain up to 120 times as much of this enzyme as extracts grown on α-alanine. Optimum conditions include a high ammonium ion concentration at high pH (in the range of pH 8.1) and a very high ionic strength (up to 9.0). The evidence suggests that the active species attacking the double bond system is free ammonia with its unshared pair of electrons rather than the charged ammonium ion. The reaction is freely reversible, although the ΔF is about -4660 calories at pH 7.5 in favor of the amination direction (71). The substrate specificity for ammonia and acrylyl CoA is absolute.

In the second paper, Vagelos, Earl & Stadtman (70) described a new enzyme acrylyl CoA hydrase, which catalyzes the hydration of acrylyl thioesters to lactyl thioesters. The enzyme occurs in both a pseudomonad grown on propionic acid as a sole source of carbon and in pigeon heart muscle extract. The reaction appears to be irreversible. No lactyl CoA dehydrogenase was detected, although a deacylase was found in *Cl. propionicum* and *Clostridium kluyveri*. The enzyme is of interest from the point of view of mechanism, since the double bond system conjugated with its thioester group would suggest a β addition rather than an α addition. The product is L(+) lactic acid on the basis of its serving as a substrate for lactic dehydrogenase.

Cell-free extracts of *Cl. kluyveri* catalyze the oxidation of propionic acid by a series of reactions peculiar to this organism (71):

$$\text{Acetyl P} + \text{propionate} + \text{CoA} \rightleftharpoons \text{propionyl CoA} + \text{acetate} + P_i \quad 22.$$

$$\text{Propionyl CoA} \rightleftharpoons \text{acrylyl CoA} + 2H \quad 23.$$

$$\text{Acrylyl CoA} + H_2O \rightleftharpoons \beta\text{-hydroxypropionyl CoA} \quad 24.$$

$$\beta\text{-Hydroxypropionyl CoA} + TPN^+ \rightleftharpoons \text{malonyl semialdehyde CoA} + TPNH + H^+ \quad 25.$$

$$\text{Malonyl semialdehyde CoA} + TPN^+ + H_2O \rightarrow \text{malonyl CoA} + TPNH + H^+ \quad 26.$$

β-Hydroxypropionyl CoA dehydrogenase was purified some fivefold from extracts of *Cl. kluyveri* (71). The enzyme does not attack free β-hydroxypropionic acid and is readily reversible. Vagelos reports that malonyl semialdehyde CoA may undergo further reactions. Thus, in *Cl. kluyveri*, a malonyl semialdehyde CoA dehydrogenase catalyses Reaction 26; in animal tissue extracts, two reactions are evident, the first is dependent on DPNH for the disappearance of malonic semialdehyde CoA, presumably to β-hydroxypropionyl CoA, and the second requires DPN^+, for further oxidation.

Thus, several systems have now been described that metabolize β-hydroxypropionyl CoA. They may be summarized as in Figure 2.

Martin & Batt (72) reported that washed cell suspensions of *Nocardia corallina* oxidize propionate. The oxidation rate is increased by CO_2. Pennington & Appleton (73) have noted the inhibition of acetate oxidation by propionate in homogenates of rat liver. The inhibition was released somewhat by increasing CoA concentration. It is possible that a competion exists be-

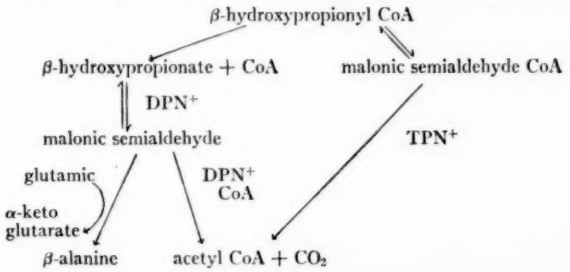


FIG. 2. Metabolic pathways of β-hydroxypropionic acid.

tween propionate and acetate for the essential cofactors such as ATP and CoA. Further, it would be of interest to examine the effects of known products of propionate oxidation, such as acrylyl CoA and β-hydroxypropionyl CoA, on the oxidation of acetate in Pennington's system. Black & Kleiber (74) intraveneously injected propionate-1-C^{14} and 2-C^{14} into lactating cows and observed that the specific activities of aspartic acid, serine, and lactose were very similar, suggesting that propionate may serve as a precursor of these two amino acids as well as for lactose. Since alanine has a low specific activity relative to serine, propionate probably followed the succinate route in the intact cow. Pomerantz (75) studied by mass analysis the metabolism of propionate by *Propionibacterium arabinosum* carbon atom 3 of (1:3-C^{14}) propionate is randomized to C-2 without loss of C^{14} from the carboxyl carbon and with no net change in the amount of propionate.

Branched chain acids.—Coon and his group (76) have continued to explore the transformations of the branched acids derived from leucine and valine. Extracts prepared from rat liver or pig heart can catalyze the following reactions:

$$\text{Isobutyryl-CoA} \rightleftharpoons \text{methacrylyl CoA} + 2H \qquad 27.$$

$$\text{Methylacrylyl CoA} + H_2O \rightleftharpoons \beta\text{-hydroxyisobutyryl CoA} \qquad 28.$$

$$\beta\text{-Hydroxyisobutyryl CoA} + H_2O \rightarrow \beta\text{-hydroxyisobutyric acid} + \text{CoA} \qquad 29.$$

$$\beta\text{-Hydroxyisobutyric acid} + DPN^+ \rightleftharpoons \text{methyl malonic semialdehyde} + DPNH \qquad 30.$$

Reaction 27 is probably catalyzed by acyl dehydrogenase, which has a normal specificity for C_4-C_8 acyl CoA derivatives. Crystalline crotonase participates in Reaction 28. Crude extracts catalyze Reactions 29 and 30. A transaminase reacts with glutamate and methyl malonic semialdehyde to form β-aminoisobutyrate. Presumably, methyl malonic semialdehyde can undergo an oxidative decarboxylation to propionyl CoA (76, 77).

β-Hydroxyisobutyric acid dehydrogenase was purified some 200-fold from pig kidney and has no detectable activity toward other hydroxy compounds, including β-hydroxybutyrate or its CoA derivatives (77). DPN^+ is the specific hydrogen acceptor. The enzyme is widespread in nature. Maximal pH activity is at pH 9.0; *Km* for hydroxyisobutyrate is $1.2\times10^{-4}M$ and $5.4\times10^{-5}M$ for DPN^+. Apparent *Keq* for the reaction, taking (H^+) into account, was 3.0×10^{-11}, indicating that the equilibrium position markedly favors methylmalonate semialdehyde reduction. Since DL-hydroxyisobutyrate was employed as substrate, no information is available as to stereospecificity of the enzyme.

Hydroxyisobutyryl CoA deacylase also hydrolyzes β-hydroxypropionyl CoA (21). It was purified about eightfold from pig heart extracts. Optimal pH is 5.6. Other possible substrates were not attacked.

The description of an unusual enzymic activation of CO_2 by Bachhawat & Coon (60), in 1957, aroused great interest in biochemical circles. On the basis of a CO_2 dependent, hydroxylamine-dependent liberation of pyrophosphate from ATP, which was catalyzed by a crystalline enzyme obtained from pig heart, Bachhawat & Coon proposed this mechanism (60):

$$CO_2 + ATP \rightleftharpoons AMP - CO_2 + PP \quad 31.$$

$$AMP - CO_2 + \beta\text{-hydroxyisovaleryl CoA} \rightarrow \beta\text{-hydroxy-}\beta\text{-glutaryl-CoA} + AMP \quad 32.$$

In 1959, a re-examination of this reaction mechanism was carried out by Kupiecki & Coon (59). They showed that the products are ADP and a non-nucleotide phosphate-containing compound, which was neither phosphate nor pyrophosphate. It was shown that a non-specific colorimetric method for pyrophosphate [Flynn, Jones & Lipmann (78)] will give a positive test. However, crystalline pyrophosphatase does not react with the phosphate-containing reaction product. Furthermore, the product is more labile to acid hydrolysis than is pyrophosphate. The compound is, on the other hand, quite stable to alkali. It has not yet been identified. Furthermore, the enzymes catalyzing the CO_2- and NH_2OH-dependent degradation of ATP do not participate in the actual carboxylation reaction.

The enzyme catalyzes the exchange of ADP^{32} with ATP, whereas P^{32} or inorganic phosphate, pyrophosphate, and C^{14}-AMP failed to exchange with ATP under the same conditions. The exchange is independent of CO_2 concentration. The inability of fluoride to substitute for NH_2OH would suggest that the activating enzyme is distinct from fluorokinase (see page 269). In a note added in proof, Kupiecki & Coon (59) stated that the ratio of activating enzyme to pyruvic kinase is fairly constant. Crystalline pyruvic kinase duplicates the activity of the activating enzyme in their standard assay system. Therefore, the zinc-dependent enzyme and the magnesium-dependent fluorokinase bear a close relationship to each other on the basis of a common pyruvic kinase activity associated with the two proteins.

Coon and his co-workers (79) recently re-examined the role of β-hydroxyisovaleryl CoA as the CO_2 acceptor. From the standpoint of organic chemistry, it is difficult to explain the participation of β-hydroxyisovaleryl CoA, since there is no means by which the γ-carbon could release a proton to form a fractional negative charge. Knappe & Lynen reported at the Fourth International Congress of Biochemistry, in Vienna (80) that an enzyme system isolated from mycobacterium catalyzed the reaction:

$$\beta\text{-Methyl crotonyl CoA} + CO_2 + ATP \rightarrow \beta\text{-methyl glutaconyl CoA} + ADP + P_i \quad 33.$$

A specific hydrase, methyl glutaconase, then hydrates the product to β-hydroxy-β-CH_3-glutaryl CoA (81). Similar results are now reported by Coon (79) with a purified chicken liver preparation. Apparently, sufficient crotonase activity was present in Coon's previous described enzyme systems to supply β-methyl crotonyl CoA from β-hydroxyisovaleryl CoA, the added substrate. By preincubating with PCMB, crotonase is inhibited: under these conditions, only β-methyl crotonyl CoA is reactive. β-Methyl crotonyl CoA is ideally suited as a CO_2 acceptor, because its double bond system is conjugated with its thioester group. The γ-carbon will now readily lose a proton and have a fractional negative charge which CO_2 can readily attack. This carboxylase had been shown to contain biotin by both Lynen (82) and Coon (83). The mechanism of biotin and CO_2 fixation is discussed on page 287.

Rudney and his group have further explored the system responsible for the synthesis of β-hydroxy-β-methyl glutaryl CoA (27, 28). They have prepared from yeast a thirtyfold purified enzyme preparation which catalyzes this reaction:

$$\text{Acetyl CoA} + \text{acetoacetyl CoA} \rightleftharpoons \beta\text{-hydroxy-}\beta\text{-methyl glutaryl CoA} + \text{CoA} \quad 34.$$

It was of interest to determine whether the thioester bond of acetoacetyl CoA or acetyl CoA was the one hydrolyzed (28, 81). Labeled β-hydroxy-β-methyl glutaryl CoA, prepared from acetyl CoA-1-C^{14} and unlabeled acetoacetyl CoA, was cleaved by the cleavage enzyme to acetoacetate and acetyl CoA. Radioactivity was found exclusively in the carboxyl group of acetoacetate, thereby proving that the thioester bond that was hydrolyzed during the condensation arose from acetyl CoA:

$$CH_3COCH_2COCoA + CH_3COCoA \underset{\text{enzyme}}{\overset{\text{condensing}}{\rightleftharpoons}} \begin{array}{l} COCoA \\ | \\ CH_2 \\ | \\ HOC{-}CH_2COOH + CoA \\ | \\ CH_3 \end{array}$$

$$\xrightarrow{\text{cleavage enzyme}} CH_3COCH_2COOH + CH_3COCoA$$

Rudney & Ferguson (28) have estimated that the total free energy changes in the condensation reaction is approximately −11,670 calories at pH 7.0 in favor of the condensation reaction. The driving force here, as also in the citrate system, is the cleavage of the thioester bond.

Ferguson, Durr & Rudney (84) and Knappe (85) in Lynen's laboratory have recently extended their investigations concerning the transformations of β-hydroxy-β-methyl glutaryl CoA by preparing an enzyme extract from yeast that, in the presence of TPNH, catalyzes the two-step reduction of β-hydroxy-β-methyl glutaryl CoA to mevalonic acid. Although mevaldic acid would be the logical intermediate in the reduction sequence, no evidence for its transient formation could be obtained. Rudney, therefore, suggested the sequence shown in Figure 3.

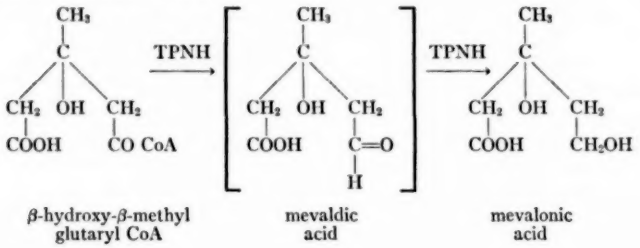


FIG. 3.

Dicarboxylic Acids

Oxalic acid.—Knowledge concerning the synthesis or breakdown of the simple dicarboxylic acids has increased considerably in the last few years. Hayaishi (86) observed in 1956 that oxalic acid is produced from oxaloacetate by an enzyme system isolated from the mycelia of *Aspergillus niger*. Oxalic acid is in turn converted to formic acid and CO_2 by an enzyme obtained from soil bacteria (87). This reaction is anaerobic and requires ATP, CoA, Mg^{++}, thiamin pyrophosphate, and acetate. Only catalytic amounts of acetate are needed; the ATP, CoA, and acetate requirements can be replaced by acetyl CoA. In the absence of thiamin pyrophosphate, oxalohydroxamate accumulates. The evidence suggests the following reactions:

$$\text{Acetate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{++}} \text{acetyl CoA} + \text{PP} + \text{AMP} \qquad 35.$$

$$\text{Acetyl CoA} + \text{oxalic} \longrightarrow \text{oxalyl CoA} + \text{acetate} \qquad 36.$$

$$\text{Oxalyl CoA} \xrightarrow{\text{ThPP}} (\text{formyl CoA}) \longrightarrow \text{formic} + CO_2 + \text{CoA} \qquad 37.$$

This system is found in various organisms.

In addition, a more novel enzyme, an oxalic acid decarboxylase, which catalyzes the following reaction, has been studied in the wood-destroying fungus *Collyvia veltipes* (88):

$$(COOH)_2 \rightarrow HCOOH + CO_2 \qquad 38.$$

The enzyme has been purified 400 times and shows no cofactor requirements, with the exception of small amounts of O_2, although the overall stoichiometry does not involve O_2 utilization. The usual chelating reagents, such as ethylenediaminetetraacetate or CN^- and α,α'-dipyridyl, had no effect, nor did flavin mononucleotide, H_2O_2, and cytochrome-*c*. Here again, rigid substrate specificity is observed.

A flavin-containing oxalic oxidase that catalyzes the reaction oxalic acid $+O_2 \rightarrow CO_2 + H_2O_2$ has been described by Finkle & Arnon (89) and Datta & Meeuse (90) in higher plants and mosses.

Malonic acid.—The metabolism of malonic acid has been well studied in recent years. Thus, in bacteria (91), plants (92, 93), and animal tissues (94), including human chorionic homogenates (95), a common mechanism has been shown.

$$\text{Malonic} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{++}} \text{malonyl CoA} + \text{PP} + \text{AMP} \qquad 39.$$

$$\text{Malonyl CoA} \longrightarrow \text{acetyl CoA} + CO_2 \qquad 40.$$

Recently, Jakoby (96) obtained from a strain of *Pseudomonas fluorescens* an enzyme which was purified 200 times and oxidatively decarboxylated malonic semialdehyde:

$$HOCCH_2COOH + DPN^+ + CoA \rightarrow CH_3CO\ CoA + CO_2 + DPNH + H^+$$

Succinic semialdehyde, acetaldehyde, and malonyl CoA are inert. The enzyme behaves like a single protein. It would be of interest to determine from what carbon in malonic semialdehyde CO_2 is derived.

Glutaric acid.—Glutarate-3-C^{14} was administered to male rats (97), and the C^{14} was found mostly in glutamate, aspartate, alanine, and respiratory CO_2. C^{14} activity was located mostly in the carboxyl position of the amino acids, indicating that the major route of breakdown must be via acetate-1-C^{14}. The proposed pathway explains these results:

$$\text{Glutarate-3-C}^{14} \rightarrow \beta\text{-hydroxy glutarate-3-C}^{14} \rightarrow \text{acetondicarboxylate-3-C}^{14} \rightarrow \text{acetoacetate-3-C}^{14} + CO_2 \rightarrow 2 \text{ acetate-1-C}^{14} \qquad 41.$$

Acetylenic acids.—Bu'Lock & Gregory (98) examined the products formed from the incorporation of acetate-1-C^{14} into the polyacetylenic acid of the fungus, *Basidiomycete* B841. Earlier, these workers (99) showed that this fungus excretes as much as 1 mg./ml. of the polyacetylenic acid into the culture media. These acids have been identified as nemotinic and odyssic acids. Since these acids are formed from an intermediate in normal glucose breakdown, it was logical to study the distribution of C^{14} activity from intermediates such as acetate-C^{14}. Total incorporation of acetate-1-C^{14} into polyacetylenes was usually 15 to 20 per cent, with the activity of the individual carbon atoms being confined to the odd-numbered atoms. Nemotinic acid has 11 carbon atoms and is presumably formed from six molecules of acetate linked head to tail with elimination of the terminal methyl group. Odyssic acid (C_{12} acid) is formed from six acetates without CH_3 elimination. Both acids are γ-hydroxy acids with two triple bonds and an allene group:

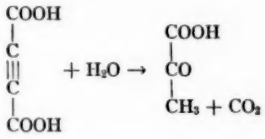
Nemotinic acid: $HC{\equiv}C{-}C{\equiv}C{-}CH{=}C{=}CHCHOHCH_2CH_2COOH$

Odyssic acid: $CH_3C{\equiv}C{-}C{\equiv}C{-}CH{=}C{=}CHCHOHCH_2CH_2COOH$

Presumably formed within the framework of the known fatty acid synthetic systems, the triple bonds may arise directly or indirectly from $-COCH_2-$ and the allene groups may be formed from either incomplete conversion into or secondary formations of the acetylenic group.

To gain more insight into the metabolism of acetylenic acids, workers have turned to the enrichment culture techniques of microbiology. Eimhjellen (100) has isolated a facultative anaerobic bacterium and identified it as a member of the family of Enterobacteriacae. The organism grows on acetylene dicarboxylic acid as the sole carbon source and ammonium N as the only nitrogen source. Acetylene dicarboxylic acid is converted by resting cell suspensions in the absence of oxygen to CO_2, lactate, succinate, acetate, formate, and ethanol. Pyruvic acid has been isolated and characterized. CO_2 output from acetylene dicarboxylic acid is completely inhibited by 2×10^{-2} *M* fluoride. More recently, Eimhjellen (101) prepared extracts from aerobically grown cells. These extracts convert acetylene dicarboxylic acid to oxaloacetate, which then undergoes further decomposition to pyruvic acid and CO_2. In extracts from anaerobically grown cells, oxaloacetate accumulates in lower yields from acetylene dicarboxylic acid. The author concludes that this organism metabolizes the acetylenic dicarboxylic acid by direct hydration of the triple bond to form oxaloacetate, which may then undergo more

conventional transformations. Yamada & Jakoby (102) incubated acetylenic dicarboxylic acid as a sole carbon source in an enrichment culture. A pseudomonad was isolated which rapidly decomposes acetylene dicarboxylic acid as shown in Reaction 42.

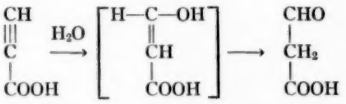


42.

Purified 150 times, the enzyme converts 140 μM of acetylene dicarboxylic acid to pyruvate per min. per mg. protein. No cofactors were formed and no indication of more that one protein was observed. The enzyme had high substrate specificity. Oxalacetate was neither a free intermediate of the reaction, nor did it serve as a substrate. An unsuccessful search was made in animal and plant tissues for enzymic activity.

A mechanism that would involve the addition of H_2O to form a transient hydroxyfumarate in equilibrium with oxalacetate and then undergo decarboxylation had no experimental support, although it is possible that this intermediate is only formed on the enzyme surface and then immediately decarboxylated to pyruvate. Although it would appear that the results of Yamada & Jakoby and Eimhjellen are in conflict, it is quite possible that two different enzyme systems may be involved.

Yamada & Jakoby (103) next studied the breakdown of propiolic acid (acetylenemonocarboxylic acid). Accordingly, a pseudomonad, with the ability to grow on propiolic acid as the sole carbon source was isolated, and an enzyme was prepared from extracts of this organism. The enzyme catalyzes the sequence shown in Reaction 43.



43.

No pyruvate is formed, and the enzyme has absolute substrate specificity. Addition of H_2O to the triple bond is the expected type of addition in α, β-triple bond carboxylic acids. These results eliminate the possibility that, in the decarboxylation of acetylene dicarboxylic acid, the first step could be a decarboxylation to propiolic acid, since malonic semialdehyde would then be the expected final product and not pyruvic acid.

Polyunsaturated Fatty Acids

According to Thomasson (104), the higher polyene acids can be separated into two different groups based on the position of their double bonds. The presence of the 1,4 double bonds toward the carboxyl end of the fatty acid molecule results in enhancement of essential fatty acid, while extension of

the 1,4 polyene system toward the methyl group causes a loss in activity. Biological potency accordingly decreases with arachidonic <linoleic <linolenic acid. A biochemical explanation for the biological activity of polyunsaturated fatty acids is still poorly understood. Considerable information is, however, available concerning the interconversions of these unsaturated acids in whole tissue

The fungus, *Trichoderium viride* readily synthesizes the unsaturated and saturated fatty acids. Crombie & Ballance (105) have studied the incorporation of acetate-C^{14} into the fatty acids of the mycelia of this fungus. With a two-day-old mycelium, incorporation of acetate in 3 hr. yielded palmitic, stearic, and oleic acids of relatively high specific activity, whereas linoleic and linolenic acids had much lower specific activities. Crombie concluded that oleic acid is the first unsaturated fatty acid to appear and dismissed the hypothesis that oleic acid is formed by a hydrogenation reaction from the more highly unsaturated fatty acids. If now the two-day-old mycelium, after being fed 3 hr. with acetate-C^{14}, is transferred to a non-radioactive medium for two additional days and the fatty acids are then analyzed, palmitic, stearic, and oleic acids show no significant decrease in specific activity, whereas linoleic and linolenic have increased severalfold. Crombie suggested that the more polyunsaturated acids are formed by successive unsaturation steps of oleic acid.

It has been known for some time that the feeding of trienoic acid (linolenic) resulted in increases in trienoic, pentaenoic, and hexaenoic acids in the tissue fats. When linoleic acid was fed, there were increases in dienoic and tetraenoic acids. When placed on a fat-free diet, the animal showed considerable increases in trienoic acid (106 to 108).

Linoleic acid transformations.—In 1953, Mead (109) noted that, when acetate-1-C^{14} was injected into a rat, the linoleic acid subsequently isolated had no appreciable C^{14} in its carboxyl carbon, whereas arachidonic acid had considerable C^{14} activity in its carboxyl carbon. This suggested that arachidonic acid is formed in the body from acetate and some C^{18} precursor. When carboxyl labeled linoleic acid was fed (110), C^{14} active arachidonic acid was isolated, reduced, and degraded to show that 74.7 per cent of the radioactivity was in the carboxyl carbon, 0.7 per cent in the α carbon atom, and 24.5 per cent in the β carbon atom. Evidently, linoleic-1-C^{14} acid is partly incorporated as a unit with a subsequent C_2 addition to form the C_{20} acid and partly degraded as C_2 fragments, which are then reincorporated to the final C_{20} acid. The entire sequence requires a chain lengthening of two carbon atoms and the removal of four hydrogen atoms to form two additional double bonds. Since Thomasson (104) has already reported that γ-linolenic acid (6, 9, 12 octadecatrienoic acid) could be postulated as an intermediate in the conversion steps, Mead (111) tested this hypothesis directly by injecting methyl-γ-linolenate-1-C^{14} into a rat and isolating the depot and organ lipids. The sole highly active fatty acid was arachidonic acid, which indicates that γ-linolenic acid was incorporated almost exclusively into the

arachidonic acid. While both palmitic and stearic acids had low activities, the monounsaturated C_{16} and C_{18} acids had even lower C^{14} activity. Mead concluded that γ-linolenic acid is not hydrogenated stepwise to stearic acid but rather that β-oxidation cleaves C_2 units from linolenic-1-C^{14}. These then are recondensed to the saturated C_{16} and C_{18} acids, which are in turn dehydrogenated to the monounsaturated acids.

The degradation of arachidic acid (derived by reduction from arachidonic acid) is indicated as:

$C_{17}H_{35}$	CH_2	CH_2	COOH
1.2	97.4	0.3	1.1

The lack of much C^{14} migration indicates the rapid conversion of γ-linolenic-1-C^{14} acid to arachidonic acid. The most probable pathway is summarized in Reaction 44.

$$\begin{array}{l} CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH \\ \quad\downarrow -2H \qquad \text{linoleic acid} \\ CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_4COOH \\ \quad\downarrow +C_2 \qquad \gamma\text{-linolenic acid} \\ CH_3(CH_2)_4(CH{=}CHCH_2)_2(CH_2)_5COOH \\ \quad\downarrow -2H \qquad \text{homo-}\gamma\text{-linolenic acid} \\ CH_3(CH_2)_4(CH{=}CHCH_2)_4{-}(CH_2)_2COOH \\ \qquad\qquad \text{arachidonic acid} \end{array} \qquad 44.$$

Ogilvie & Langdon (112) have observed the formation of a long chain olefinic acid when mevalonic acid is incubated with an enzyme preparation from homogenates of rat liver. The structure of the acid has been elucidated by Ogilvie (113) as a branched chain polyunsaturated terpenoid monocarboxylic acid.

In 1952, Witten & Holman (114) submitted evidence that pyridoxine is necessary for the conversion of linoleic acid to arachidonic acid in rats. When young rats were placed in a fat-deficient, pyridoxine-deficient diet, definite symptoms of acrodynia appeared after four weeks. Pyridoxine plus linoleate relieved these symptoms and stimulated growth, fat synthesis, and arachidonate synthesis much more than either supplement alone. This would imply that the pyridoxine-deficient animal may suffer from a secondary deficiency of arachidonic acid. However Williams & Hincenbergs (115) reported that, when pyridoxine-deficient rats were fed methyl arachidonate, the acrodynia of the animal was not relieved. They concluded that arachidonic acid and linoleic acid are not directly implicated in the acrodynia of the deficient animals.

Bernhard *et al.* (116) fed to unsaturated fatty acid-deficient rats C^{14}-oleic, linoleic, and γ-linolenic acids. The activity of expired CO_2 showed, in 24 hr., 46.7 per cent oxidation of C^{14}-oleic, 38 per cent linoleic, and 65 per cent γ-linolenic acids. In normal rats, no significant differences in oxidation

rates compared to the deficient rats were observed. Thus, the capacity of the cells to oxidize the polyunsaturated fatty acids remains the same regardless of the nutritional background.

Lynn & Brown (117) reported the preparation of a rat liver mitochondria that readily oxidized linoleate and linolenate (as measured by $C^{14}O_2$ formation from carboxyl labeled fatty acids). An essential cofactor is DPNH. Extracts of acetone powders of these mitochondria require CoA, ATP, and DPNH for maximum oxidation. It would be of considerable interest to determine the intermediates of oxidation of these acids particularly because of the DPNH requirement. There is little evidence at present to suggest a hydrogenation of unsaturated acids to the saturated acids.

Lipid fractions of the human red cell (including phospholipid, lipoproteins, and triglycerides) have been studied by James *et al.* (118). When acetate-C^{14} is incubated with human red cells, C^{14} linoleic acid and arachidonic acid are isolated from the total lipid. Since labeling was uniformly distributed over all the hydrocarbon chain of these acids, *de novo* synthesis is indicated. This system may prove useful as an *in vitro* system to study the synthesis of these polyunsaturated acids.

Linolenic acid.—When methyl linolenate-1-C^{14} is fed to a rat, the higher unsaturated fatty acids acquire C^{14} activity (119). Linolenic acid is presumably converted to C_{20} (tetraenoic acid, which is isomeric with arachidonic acid) and C_{22} pentenoic acids. There is little or no conversion of linolenic to linoleic acid.

The distribution of activity in arachidic acid (derived by hydrogenation of the C_{20} fraction) is as follows:

$C_{16}H_{33}$—	CH_2—	CH_2—	CH_2	COOH
1.0	3.8	60.2	7.8	27.2

The activity in C_1 probably originated from a C_2 cleavage of linolenic-1-C^{14} and a recondensation to the C_{20} acid.

Oleic acid.—Fatty acids were isolated from the liver and carcass of rats killed 8 hr. after feeding C^{14}-oleic or 24 hr. after feeding C^{14} linoleic or C^{14} linolenic acid (120). The unsaturated fatty acids contain 91 to 98 per cent of the total radioactivity of fatty acids. Since palmitic and stearic acids had low activity and the same order of magnitude as cholesterol, Bernhard *et al.* (120) suggested that the saturated acids originated from C^{14}-acetate rather than directly by hydrogenation of unsaturated fatty acids. When stearic-C^{14} was fed, the following percentages of radioactivity were obtained: stearic 57 per cent, palmitic 15.5 per cent, and oleic 27.5 per cent, which would suggest dehydrogenation of stearic to oleic acid.

When rats are maintained on a fat-deficient diet, there is a sharp increase in the trienoic acids in the tissues, the main component being 5,8,11-eicosatrienoic acid. Holman *et al.* (121) has suggested that oleic acid may serve as a potential precursor and may be modified by the addition of a C_2 unit and the removal of four hydrogens. Fulco & Mead (122) tested this possible route

by injecting into rats acetate-1-C^{14} and then isolating from the depot and body lipids oleic and eicosatrienoic acids. The unsaturated acids were then either submitted to permanganate oxidation or to catalytic reduction and subsequent degradation to margaric acid. On the basis of the distribution of C^{14} activity in the mono- and dicarboxlyic acids derived from permanganate oxidation and from the stepwise degradation of the saturated C_{18} and C_{20} acids, Fulco & Mead (122) demonstrated that oleic acid C^{14} distribution was strikingly similar to that of 5,8,11-eicosatrienoic acid as shown in Figure 4 in which the numerals indicate the percentage radioactivity.

Fulco & Mead (122) postulate the following reactions in the transformation of oleic acid to 5,8,11-eicosatrienoic acid.

$$\begin{array}{l} CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH \\ \quad -2H \;\big|\; \text{oleic acid} \\ \downarrow \\ CH_3(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4COOH \\ \quad +2C \;\big|\; \text{6,9-octadecatrienoic acid} \\ \downarrow \\ CH_3(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_6COOH \\ \quad -2H \;\big|\; \text{8,11 eicosadeinoic acid} \\ \downarrow \\ CH_3(CH_2)_7CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3COOH \\ \quad \text{5,8,11-eicosatrienoic acid} \end{array} \qquad 45.$$

Since the results were obtained from feeding experiments with intact animals, nothing can be said concerning the requirement for CoA and other cofactors. Undoubtedly, in the near future many questions concerning the detailed mechanisms of these reactions will be explored and elucidated.

A different approach to the study of the mechanisms of unsaturation has been reported by Bloomfield & Bloch (123). They grew a special strain of *Saccharomyces cerevisiae* LK2G12 under relatively anaerobic conditions. Extracts prepared from the cells were centrifuged at 104,000 x g to a clear particle-free supernate and the residue. When both fractions were dialyzed and recombined, added palmitic acid was converted to palmitoleic acid in the presence of TPNH, a divalent cation, and oxygen. DPNH was less active. Bloch reported recently (124) that palmityl CoA is the actual substrate. The

$$\begin{array}{l} \overbrace{\underbrace{CH_3(CH_2)_7CH}_{77.9}}^{39.1} = \overbrace{\underbrace{CH(CH_2)_7COOH}_{22.1}}^{60.9} \\ \qquad\qquad \text{Oleic Acid} \\ \overbrace{\underbrace{CH_3(CH_2)_7CH}_{77.2}}^{36.6} = \overbrace{\underbrace{CH(CH_2{-}CH{=}CH)_2CH_2CH_2}_{22.8}}^{63.4} \\ \qquad\qquad \text{5,8,11-eicosatrienoic acid} \end{array}$$

FIG. 4. Comparison of the distribution of radioactivity amongst the carbon atoms of oleic and 5,8-11-eicosatrienoic acid.

reaction strongly resembles an oxygenase in that it probably involves TPNH and O_2:

$$R^1CH_2CH_2R^2 \xrightarrow[O_2]{TPNH} R^1CHOHCH_2R^2 \xrightarrow{-H_2O} R^1CH{=}CHR^2 \qquad 46.$$

Bernhard *et al.* (125) have observed that rat liver mitochondria are inactive in dehydrogenating stearic acid to oleic. However, with rat liver supernatant free of mitochondria, stearate-1-C^{14} is converted to an acid which on periodate oxidation yields radioactive azelaic acid. Presumably, the formation of oleic acid is dependent on ATP. No detailed cofactor studies are reported

In an interesting study, Tappel & Zalkin (126) have correlated oxygen absorption of isolated rat liver mitochondria with the appearance of lipid peroxide. With the rise of lipid peroxide, an increased deterioration of DPNH cytochrome reductase and succinic oxidase was observed. Presumably, polyunsaturated fatty acids in mitochondria undergo hematin-catalyzed peroxidations. This can be inhibited by suitable concentration of α-tocopherol or butylated hydroxyanisole.

Fatty Acid Synthesis

Studies have continued on fatty acid interconversion by lactobacilli (127), metabolism of 3,3, dimethyl myristic acid (128), and acetate-C^{14} incorporation into various tissues (123 to 147).

This past year has seen the development of a new concept to explain the multiple condensation of C_2 units and plausible mechanism of biotin participation in fatty acid synthesis.

With the development of the detailed reactions of the β-oxidation sequence, workers in the field accepted the reasonable hypothesis that synthesis was simply a reversal of β-oxidation. Stansley & Beinert (148) were the first to test this hypothesis with highly purified enzymes of the fatty acid β-oxidation cycle, reduced benzylviologen as the reductant, and acetyl CoA. Only comparatively small amounts of butyryl CoA were synthesized. No higher homologues accumulated. This failure was attributed to an unfavorable *Keg* of the thiolase reaction which greatly favors breakdown. The discovery by Langdon (149) of the TPN^+-ethylene reductase, which apparently was required for the reduction of double bond systems to saturated acyl CoA derivatives, was at first believed to explain the lack of success by the earlier workers in their attempts to reverse the fatty acid spiral. Earlier, in 1952, Brady & Gurin (150) first described a pigeon liver extract system that incorporated acetate-C^{14} into long chain fatty acids. A particle-free supernatant, when reinforced with a water-soluble extract of acetone-dried mitochondria, readily synthesized long chain fatty acids but only when supplemented with Mg^{++}, DPN^+, ATP, and CoA. Free fatty acids were the primary products. No TPN^+ requirement was observed. Citrate markedly stimulated synthesis. A similar system was reported by Popják & Tietz (151) in 1954.

Supernatant fractions of lactating rat mammary gland incorporated acetate-C^{14} into short chain fatty acids (C_4–C_8). Crude homogenates could synthesize C_8–C_{18} acids. While oxaloacetate or α-ketoglutarate stimulated incorporation, malonate markedly increased synthesis. ATP, CoA, and DPN^+ were also required. Hele *et al.* (152, 153) in 1957 described the preparation of a supernatant solution from an homogenate of lactating rabbit mammary glands, which synthesized short chain fatty acids. Again ATP, CoA, Mg^{++}, cysteine, and DPNH, but not TPNH, were required for the synthesis of acyl hydroxamates. CoA derivatives were later detected in this system. In 1958, Lachance *et al.* (154) obtained evidence suggesting that the microsomes served as important reductive sites for fat synthesis. When microsomes were added to an extract of lactating rabbit mammary glands and acetate-C^{14}, TPNH, CoA, and ATP, or crotonyl CoA-C^{14} and TPNH, butyryl CoA was formed at the expense of TPNH. In 1957 Seubert, Greull & Lynen (155) prepared a TPNH-ethylene reductase from pig liver mitochondria, which, when added to highly purified enzymes of the β-oxidative system, catalyzed the conversion of hex-2-enoyl CoA+acetyl CoA-C^{14} to fatty acids with chain lengths up to C_{16}.

In the same year, Stumpf & Barber (156) prepared particles from avocado fruit mesocarp that readily incorporated acetate into esterified long chain fatty acids. ATP, CoA, and Mn^{++} were essential components. No clear-cut requirement could be demonstrated for TPN^+ or DPN^+.

In the meantime, the Wisconsin group led by Wakil (157) decided to submit the pigeon liver system to vigorous purification rather than attempt to reverse the β-oxidation cycle by varying conditions. From homogenates of pigeon liver, four fractions [R_1 (0 to 25 per cent), R_2 (25 to 40), R_3 (40 to 50), and R_4 (50 to 65 per cent)] were obtained by ammonium sulfate precipitation. Although the fractions alone were inactive, a combination of R_1, $+R_2+R_4$ with the required cofactors did catalyze the synthesis of long chain fatty acids. Each fraction was resubmitted to further purification and R_1, R_{23}, R_{43}, and R_{46} were obtained. The pH optimum was very sharp at pH 6.5. Tris buffer was found strongly inhibitory while phosphate and histidine buffers proved satisfactory. About a total fiftyfold purification was achieved, the best preparation incorporating 1 to 2 μM of acetate into fatty acid per mg. of total protein in 2 hr. at 38°C. Of interest is the use of 0.04 *M* $KHCO_3$ buffer in the preparation of the enzyme systems.

In 1957, Porter *et al.* (158) described the cofactor requirements of the synthesizing system. ATP, CoA, DPNH, TPN^+, Mn^{++}, isocitrate, and a sulfhydryl compound were required for full activity. CoA and ATP concentrations were critical; above 10^{-2} *M* ATP and above 10^{-4} *M* CoA, considerable inhibition occurs. DPNH was also an essential component. Isocitrate could not be replaced by a TPNH-generating system, but the TPNH-generating system is completely replaced by α-ketoglutaric acid and $KHCO_3$. Porter (159) postulated that this indicated resynthesis of isocitrate from α-ketoglutaric acid in the presence of $KHCO_3$; it is of interest to point out that

the addition of $KHCO_3$ to this system did not directly replace isocitrate. No short chain acids accumulate as possible intermediates in the reaction mixture. Small quantities of esterfied long chain fatty acids as thioesters do accumulate, but a deacylase in the system converts the newly synthesized long chain fatty acid CoA derivatives to their free acids. Of the long chain acids synthesized, 50 to 60 per cent are palmitic, 20 to 30 per cent myristic, about 10 per cent lauric, and the remainder capric, stearic, and oleic (160). The specific activity of the carboxyl carbon of fatty acids synthesized from carboxyl labeled acetate was 1.7 times that of the average carbon atom in the entire chain, therefore indicating a *de novo* synthesis of acetate-1-C^{14} units.

Tietz described a similar synthesis in preparations of chicken liver (161). When the complete homogenate was incubated with acetate-1-C^{14} in the presence of ATP, glucose-1-phosphate, isocitrate, and Mn^{++}, no fatty acids were synthesized. If now the particulate components were spun down and discarded, the supernatant catalyzed the incorporation of 0.46 μM of acetate into long chain fatty acids per 10 mg. protein for 2 hr. When submitted to fractionation procedures similar to those employed with pigeon liver supernatant solution, four fractions were obtained, R_{11}, R_{22}, R_{42}, and R_{44}. The combined fraction showed a tenfold purification over the initial crude supernatant solution. Cofactor studies indicated results very similar to those observed in the pigeon system, namely isocitrate, ATP, CoA, DPN, TPN, and $MnCl_2$ requirements. Glucose-1-phosphate also was required, although a combination of DPNH and ATP replaced it. Of the fatty acids synthesized, 80 per cent were palmitic, 17 per cent myristic, and the remainder lauric and decanoic acid (161).

In 1957, Klein (162) made a most interesting observation. He reported that with yeast homogenates, acetate was diverted to non-saponifiable fractions in absence of CO_2, but in the presence of CO_2 a fivefold increase in fat synthesis occured. No CO_2 fixation was observed in any of the lipid fractions. As early as 1948, Bloch (163) observed a stimulating effect of oxaloacetate on the synthesis of fatty acids from acetate by liver slices in a phosphate buffer. In 1950, working with liver slices, Brady & Gurin (164) reported that conversion of octanoate to long chain fatty acids occurs readily in bicarbonate buffer but not in phosphate buffer. In 1955, Lyon, Geyer & Marshall (165) noted that liver slices from fed rats synthesized long chain fatty acid from octanoate-1-C^{14} seven to eight times more when incubated in bicarbonate buffer than in phosphate buffer. In 1958, Gibson *et al.* (166, 167) observed that a highly purified system obtained from chicken liver converted acetyl CoA to palmitate in the presence of TPN^+, Mn^{++}, ATP, and bicarbonate ions. $C^{14}O_2$ is not incorporated into the long chain fatty acids but apparently played a catalytic role. Squires, Stumpf & Schmid (168) observed similar results with aqueous extracts of acetone-dried avocado mitochondria in the presence of TPN^+, ATP, Mn^{++}, and HCO_3. Wakil (166, 169) next presented evidence that biotin participates in fat synthesis. When acetyl CoA was substituted for CoA and acetate, only fractions R_1, R_2, and Mn^{++}, ATP, and

TPNH were required. Evidently R_4 contained acetothiokinase. Fraction R_1 contained a biotin concentration equivalent to about one mole of biotin per 10^6 gm. protein. Two hundred μg. of avidin greatly suppresses the incorporation of acetyl CoA into palmitic acid, indicating a functional participation of biotin. The purified fractions R_{1G} and R_{2G} (gel adsorption fractionate), contained little or no fatty acid oxidation enzymes. Thus, acyl CoA dehydrogenases, the electron-transferring fraction, thiolase, acetic thiokinase, enoyl hydrase, β-hydroxyl acyl dehydrogenase, and TPNH ethylene reductase were absent or in very low activity.

Bicarbonate is essential in both the crude supernatant solutions used earlier and in the highly purified fractions. $H^{14}CO_3^-$ is not incorporated in the fatty acids. These results strongly imply a markedly different system for fatty acid synthesis from that described by Seubert *et al.* (155).

Wakil *et al.* (170) have described a spectrophotometric assay for the synthesis of long chain fatty acids from acetyl CoA that depends upon the oxidation of TPNH in the presence of ATP, Mn^{++}, HCO_3^-, and acetyl CoA. Omission of any of these components results in little, if any, change in 340 mμ absorption. DPNH is also oxidized but much more slowly. This assay method greatly facilitates experimentation with this system.

The stoichiometry of the synthesis system followed the reaction:

$$8\ \text{Acetyl CoA} + 16\ \text{TPNH} + 16\ \text{ATP} \xrightarrow[R_{1G}+R_{2G}]{Mn^{++};\ HCO_3^-} \text{palmitate} + 16\ \text{TPN}^+ + 16\ \text{ADP} + 16\ P_i + 8\ \text{CoA} \qquad 47.$$

ATP is not used for regeneration of acetyl CoA since thiokinase is absent; its role is not known.

In an important communication, Wakil (171) was able to purify the fractions R_{1G} and R_{2G} by ion exchange chromotography to such an extent that a stepwise synthesis of fatty acids could be demonstrated. When R_{1GC} (purified by gel and column chromatography) was incubated with acetyl CoA, Mn^{++}, ATP, and HCO_3^- and the reaction mixture boiled, a compound was formed, which, when added to the R_{2GC} fraction, was quantitatively converted to long chain fatty acids. On the basis of paper chromatography and isotopic evidence, the compound was identified as malonyl CoA. Wakil concluded that the first step in fatty acid synthesis is the carboxylation of acetyl CoA to malonyl CoA. The reaction is catalyzed by an enzyme associated with the biotin-containing R_{1GC} fraction. Free malonic acid is not an intermediate. It is of interest that, in 1954, Popják & Tietz (151) reported that malonate stimulated the system obtained from mammary glands.

Brady (172) reached somewhat the same conclusions in 1958 with pigeon liver enzymes. He presented evidence that acetaldehyde condenses with malonyl CoA to yield fatty acids of longer chain lengths. Acetyl CoA did not replace malonyl CoA. Since Brady does not report the synthesizing capacity of a reaction mixture containing malonyl CoA and acetyl CoA, it is difficult

to assess the importance of acetaldehyde as a substrate in his experiments. Brady postulates that acetyl CoA is first reduced by TPNH to acetaldehyde, which then becomes the functioning C_2 unit. He cites as evidence the disappearance of TPNH when acetyl CoA is added to Fraction I of his pigeon liver enzyme. No chemical evidence is presented to demonstrate the formation of acetaldehyde. In another experiment, when malonyl CoA is added, a sharp drop in TPNH occurs in the system containing only acetyl CoA. Although Brady concluded that this result demonstrates the reduction of acetyl CoA to acetaldehyde prior to condensation with malonyl CoA, it can equally well be interpreted as a reductive step in Wakil's scheme, namely:

$$\text{Acetyl CoA} + \text{malonyl CoA} \xrightarrow{\text{TPNH}} CO_2 + \text{butyryl CoA} \qquad 48.$$

Formica & Brady (173) have also studied the primary carboxylation of acetyl CoA to malonyl CoA in extracts of pig heart. The synthesis is similar to Wakil's, requiring ATP, acetyl CoA, and Mg^{++}. No avidin effect was reported. Wakil (174) recently has presented data to support his new scheme for fatty acid synthesis. Thus, the first step in fatty acid synthesis appears to be:

$$ATP + CO_2 + \text{acetyl CoA} \xrightarrow[\text{enzyme } R_{1GC}]{Mn^{++};\ \text{biotin}} ADP + P_i + \text{malonyl CoA} \qquad 49.$$

Malonyl CoA is readily converted to palmitate by R_{2GC} in the presence of TPNH and acetyl CoA. Unlabeled acetaldehyde does not reduce the amount of C^{14}-acetyl CoA incorporated into palmitate, and there is no evidence that acetaldehyde is formed by enzymatic reduction of acetyl CoA and TPNH. C^{14}-butyryl and C^{14}-octanonyl CoA can also be incorporated into palmitate in presence of malonyl CoA. R_{2GC} does not contain any enoyl hydrase, β-hydroxyacyl dehydrogenase, thiolase, or TPNH-ethylene reduction. The evidence strongly supports the reaction sequence:

$$CH_3COCoA + CO_2 + ATP \xrightarrow{\text{biotin, } R_{1GC}} HOOCCH_2CO\ CoA \qquad 50.$$

$$\begin{aligned} CH_3CO\ CoA + COOHCH_2CO\ CoA &\xrightarrow{R_{2GC}} [CH_3COCH(COOH)CO\ CoA \xrightarrow{\text{TPNH}} \\ CH_3CHOHCH(COOH)CO\ CoA &\xrightarrow{-H_2O} CH_3CH{=}C(COOH)CO\ CoA \xrightarrow{\text{TPNH}} \\ CH_3CH_2CH(COOH)CO\ CoA] &\xrightarrow{-CO_2} CH_3CH_2CH_2CO\ CoA, \text{ etc.} \end{aligned} \qquad 51.$$

Acyl CoA will then again participate with malonyl CoA in another C_2 addition as depicted above. Thus butyryl CoA-1-C^{14} will be incorporated intact into palmitate. Long & Porter (175) have already cited evidence that butyryl CoA is incorporated into fatty acids as a unit and is thus a true intermediate.

Lynen (176) has obtained similar results with cell-free yeast extracts. ATP, Mn^{++}, CoA, and bicarbonate are required. Lynen postulates that Reactions 52 and 53 describe fatty acid synthesis. In contrast to Titchener's findings (170) only 8 ATP instead of 16 are required for the total synthesis of palmitic acid:

$$8\ CH_3CO\ CoA + 8\ CO_2 + 8\ ATP \rightarrow 8\ \text{malonyl CoA} + ADP + 8\ P_i \quad 52.$$

$$8\ \text{malonyl CoA} + 14\ \underset{(TPNH)}{DPNH} \rightarrow CH_3(CH_2)_{14}CO\ CoA + 8\ CO_2 + 7\ CoA + 14\ DPN + (TPN) \quad 53.$$

Water-clear extracts of acetone powder of avocado mitochondria require similar conditions (177). The plant system is also very effectively inhibited by as little as 10 γ of avidin. Kurtz & Miramon (178) have observed an *in vivo* biotin effect on the synthesis of polyunsaturated fatty acid by employing 16-day-old flax fruit and acetate-1-C^{14}.

An interesting variant to the malonyl CoA–CO_2 system is described by Vagelos (179). Enzyme preparations of *Cl. kluyveri* catalyze an exchange reaction between malonyl CoA and $HC^{14}O_3^-$. Malonyl CoA is not decarboxylated, and C^{14}-acetyl CoA is not incorporated into malonyl CoA. Caproyl CoA is an essential component of the system. Evidently the *Cl. kluyveri* enzyme catalyzes a reversible condensation between malonyl CoA and caproyl CoA coupled with a reversible decarboxylation: malonyl CoA+caproyl CoA$\rightleftharpoons\beta$ keto-octonyl CoA+CO_2+CoA. It is conceivable that the formation of malonyl CoA by this reaction is a mechanism for the synthesis of fatty acids via an avidin-insensitive non-biotin system. Thus, if β-keto-acyl CoA can be derived from a thiolase reaction, the reverse of the foregoing reaction would readily supply malonyl CoA for synthetic purposes.

Role of biotin in fatty acid synthesis.—It has been amply demonstrated that biotin is required in the preliminary step of fatty acid synthesis, namely, the carboxylation of acetyl CoA to malonyl CoA. Lynen and his co-workers (180) investigated a similar reaction, namely, the carboxylation of β-methyl crotonyl CoA to β-methyl glutaconyl CoA by a mycobacterium extract. They showed that the specific activity of the carboxylase could be correlated with biotin content of the enzyme protein. As would be expected, biotin could not be removed reversibly from the protein, since biotin is bound to proteins by a peptide linkage. Avidin strongly inhibits the carboxylation reaction, giving added support to the functional properties of biotin. The mechanism of the biotin effect was then studied in some detail: (*a*) When purified carboxylase is incubated with ATP and P_i^{32}, ATP^{32} can be isolated; this exchange is strongly inhibited by avidin. (*b*) CO_2 strongly inhibits P_i^{32} exchange with ATP. (*c*) When 1,3,5-C^{14} β-methyl glutaconyl CoA is incubated with carboxylase and non-labeled β-methyl crotonyl CoA, β-methyl crotonyl CoA becomes labeled. (*d*) When β-methyl glutaconyl CoA, β-methyl crotonyl CoA, Mg^{++}, and CO_2^{14} are incubated with carboxylase, C^{14} fixation is only observed when ADP is added; P_i does not replace ADP. These results can be explained by the following sequence of reactions:

$$ATP + \text{biotin—enzyme} \rightleftharpoons ADP\text{—biotin—enzyme} + P \quad 54.$$

$$ADP\text{—biotin—enzyme} + CO_2 \rightleftharpoons CO_2\text{—biotin—enzyme} + ADP \quad 55.$$

$$CO_2\text{—biotin—enzyme} + \beta\text{-methyl crotonyl CoA} \rightleftharpoons \text{biotin—enzyme} + \beta\text{-methyl glutaconyl CoA.} \quad 56.$$

If D-biotin were now substituted at a substrate level for the natural acceptor, β-methyl crotonyl CoA, in Reaction 56, Lynen noted that CO_2 would

carboxylate biotin. The reaction was followed by measuring the appearance of ADP with the ADP-pyruvic kinase-lactic dehydrogenase-DPNH assay system. L-biotin was ineffective. The $C^{14}O_2$ bound complex was very labile. Strong acid immediately released $C^{14}O_2$. In 25 min. at pH and 0°, 97 per cent of the bound $C^{14}O_2$ was released as $C^{14}O_2$. In 0.03 KOH at 0°, only 7 per cent was destroyed. The crude reaction product was dried directly and treated with diazomethane to yield a stable methylester. The product was chromatographed and identified as the methylester of biotin with CO_2 attached presumably to a nitrogen of the ureido group of biotin. Neither P_i nor adenine was found associated with the methyl ester. The structure was proven by treating biotin methyl ester with methyl chloroformate to yield the N-carbomethoxy-biotin methyl ester. When the synthetic ester was crystallized several times with the enzymatically prepared compound containing C^{14}, no change in specific activity was observed, proving that the natural ester was indeed that shown in Figure 5, and they suggest that the ADP-biotin complex probably participates with CO_2 (Figure 6).

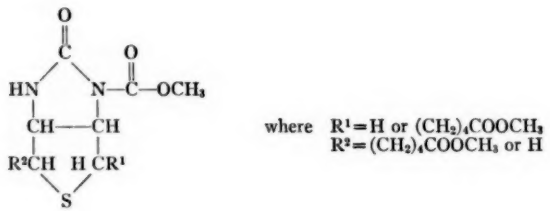


FIG. 5.

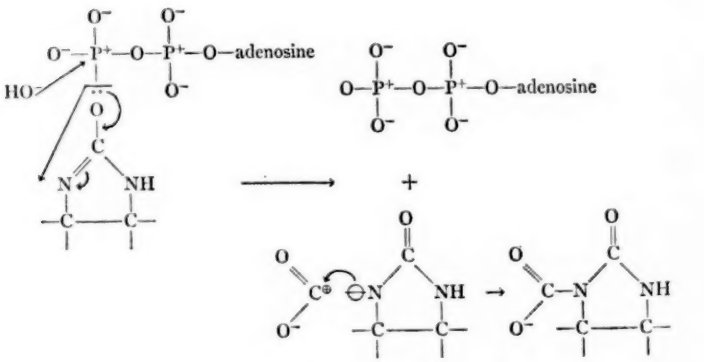


FIG. 6.

Precisely the same activation steps are catalyzed by Wakil's R_{IGC} fraction. The accepter is acetyl CoA, and the product is malonyl CoA.

Control of synthesis.—For some years it has been observed that in fasted rats hepatic lipogenesis is greatly depressed, and this can be largely overcome by oral administration of glucose to the animals before sacrifice (181, 182). Miller & Cooper (183) have studied some of the factors that control the rate of incorporation of acetate into lipids in adipose tissue. They reported that sera from rabbit, dog, and human greatly stimulate incorporation. Dialyzed sera are ineffective. However, addition of glucose, bicarbonate, and Krebs cycle amino acids restores activity to dialyzed sera.

Catravas & Anker (184) have isolated a substance from normal livers which greatly increases the rate of fatty acid synthesis in cell-free preparations from livers of fasted rats. This substance can also be obtained from yeast. The material is unstable to acid (1 N HCl) or alkaline treatment (pH 9.5), and is strongly adsorbed on charcoal. It was purified 400 times by passage through Dowex 1-formate and Dowex 50-NH_4^+, suggesting a lack of free acidic or basic groups. The concentration of the substance appears to be related to the nutritional status of the animal, since fed rats and not fasted rats yield the substance. In a more recent paper, Catravas & Anker (185) have extensively purified the factor from yeast and normal liver. As little as 6 to 10 μg. of the substance (named lipogenin), when added to a gram of liver homogenate, gives maximal synthesizing activity. Female fasted rats injected intraperitoneally with 1 mg. of lipogenin increased the ratio of acetate incorporation in injected fasted to fasted controls 5 to 1. No effects were observed on injection of the same amount of lipogenin into fed rats. Similar results are obtained with liver homogenates of fed rats. The authors suggest that this substance plays an important role in the control of fat synthesis in animals. The identification of this factor and its role in fatty acid synthesis may prove to be of great importance in lipid metabolism.

Conversion of fats into carbohydrates.—Kornberg & Beevers (186) have obtained evidence that the glyoxylate cycle is operative in providing a link between fatty acid degradation and carbohydrate synthesis during the conversion of fat into carbohydrate in castor bean endosperms. The pathway of such a conversion may be represented by the following stages:

$$\text{Fatty acid} + \text{CoA} \rightarrow \text{acetyl CoA} \rightarrow (\text{glyoxylate cycle}) \rightarrow \text{malate} \rightarrow \text{phosphoenolpyruvate} \rightarrow \text{triose phosphate} \rightarrow \text{hexose} \quad 57.$$

Beevers (187) has shown from experiments in which acetate-1 and -2-C^{14} were fed to slices of castor bean endosperms that the distribution of C^{14} within the glucose moiety of sucrose was consistent with the operation of this pathway. In addition, Kornberg & Beevers (186) demonstrated that the characteristic enzymes of the glyoxylate cycle, isocitritase, and malic synthetase, were present in cell-free extracts from the same castor bean tissue. Aconitase, condensing enzyme, and malic dehydrogenase, which participate in both tricarboxylic acid cycle variants, were also present in these enzyme preparations.

Similar studies have been carried out by Bradbeer & Stumpf (188), who used excised cotyledons from etiolated peanut and sunflower seedlings at the

physiological stage in which fat was being rapidly converted into carbohydrate. Acetate-1 and-2-C^{14} were fed to the intact cotyledons, and the distributions of C^{14} that appeared in malate and the glucose moiety of sucrose were consistent with the pathway proposed above for the conversion of fat into carbohydrate. From 3-hr. feeding experiments, carboxyl labeled acetate gave rise to malate, which was labeled virtually exclusively in the carboxyl groups, and to hexose, which had 75 to 80 per cent of its label in carbon atoms 3 and 4. In contrast, malate, with approximately 80 per cent of its label in the two middle carbon atoms, and hexose, labeled predominantly (over 90 per cent) in carbon atoms 1, 2, 5, and 6, were obtained from methyl labeled acetate. Isocitritase and malic synthetase, together with fumarase and condensing enzyme, were demonstrated in cell-free extracts from the peanut and sunflower cotyledons (188). In addition, the degradation of the radioactive malate, formed from carboxyl labeled acetyl CoA and either glyoxylate or isocitrate by these enzyme preparations, provide further confirmation that the incorporation of acetyl CoA into malate was via the malic synthetase reaction.

Carpenter & Beevers (189) have studied the distribution of isocitritase in a wide variety of plant tissues, including leaves, roots, tubers, seedlings, and fruits. They found that the only tissues that possess appreciable isocitritase activity are those in which active fat catabolism is taking place. Thus, this enzyme was demonstrated in the seedlings of castor bean, pumpkin, cottonseed, peanut, flax, sesame, soybean, watermelon, and sunflower. There is no evidence for the glyoxylate by-pass in animal tissue (190).

In studies with slices of mammary glands of pregnant, lactating, and weanling rats, Abraham & Chaikoff (191) studied the rate of conversion of glucose-1-C^{14} and glucose-6-C^{14} to CO_2 and non-volatile fatty acids. The rate appears to be higher in lactating than in non-lactating rats. The hexose monophosphate shunt does not participate in the conversion of glucose→ fatty acids in mammary gland slices from pregnant rats but does from postpartum rats. When lactation is arrested by weaning, the hexose monophosphate shunt participation ceases abruptly. Abraham & Chaikoff suggest that glucose metabolism and lipogenesis are linked through TPN^+.

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CARBOHYDRATE METABOLISM[1,2]

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The task of writing a comprehensive review on carbohydrate metabolism becomes increasingly difficult as the subject continues to expand and develop in so many of its aspects. The authors found that, in order to remain within the allotted space, certain limitations had to be introduced. Therefore, with few exceptions, preliminary notes without descriptions of experimental procedure and full detailed results have been omitted. The reviewers consider this a justified, and possibly desirable, omission as any preliminary note of interest can be expected to be followed by a complete publication and will, therefore, be better reviewed at some future date. The subject of carbohydrate transport and tissue permeability has not been reviewed because, although a number of publications have appeared on this topic, the authors do not consider that any fundamental advance in the understanding of the problem has been made in the period covered by this literature survey. No reference to clinical studies on carbohydrate metabolism and very little reference to work of a purely enzymological nature has been included, as these topics are likely to be dealt with elsewhere in this volume.

Polysaccharides and Disaccharides

Starch.—Walker & Whelan (1) have reported the synthesis of an amylose-like material, previously thought to occur only by the action of phosphorylase on α-glucose-1-phosphate, by a system containing the potato-D-enzyme. This enzyme, first described by Peat, Whelan & Rees (2), transfers two or more glucose units from maltodextrins to a saccharide molecule forming α-(1→4) glycosidic linkages. It has been shown (1) that, when the D-enzyme was incubated with maltodextrin in the presence of glucose, the average length of the polymers formed was low because of the transfer of some glucose residues to the free glucose; when, however, the glucose was converted to glucose-6-phosphate by addition of hexokinase and ATP, the high molecular polymer amylose was obtained. Peat, Turvey & Jones (3) have now described a method of purification of the D-enzyme and of the Q-branching enzyme [forming α-(1→6) glycosidic linkages] from potato juice.

[1] This review covers approximately the period from September, 1958, through August, 1959.

[2] The following abbreviations are used: DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); TPNH for triphosphopyridine nucleotide (reduced form); UDP for uridine diphosphate.

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McConnell, Mitra & Perlin (4) have studied the biosynthesis of the linear polymer, amylose, and the highly branched polymer amylopectin by administering glucose-1-^{14}C, acetate-1-^{14}C, or acetate-2-^{14}C to wheat plants and by isolating the starches from the kernels at different periods of active growth under field conditions. Measurements of the specific activities of the starch fractions at different time periods indicated that once the starch was deposited in the kernel it was not subject to extensive metabolic turnover. The specific activities of the different starch fractions were found to vary considerably. The results are in accordance with the current theory that amylopectin is formed from amylose, but the authors do not consider the data conclusive and discuss other possible pathways of amylopectin synthesis.

A study of the starch of holotrich protozoa from sheep rumen has been reported by Mould & Thomas (5). Degradation with the debranching R-enzyme (which hydrolyses the (1→6)-glycosidic branch linkage of amylopectin), α-amylase, and β-amylase has confirmed the findings of Forsyth & Hirst (6) that the polysaccharide formed by these organisms is an amylopectin. The presence of two species specific α-amylases was demonstrated by use of zone electrophoresis. The protozoa were shown to have a phosphorolytic mechanism for the synthesis of starch similar to that found in higher plants and animals.

An interesting paper has been published by Maclachlan & Porter (7) in which they demonstrated the synthesis of starch and sucrose from glucose in tobacco-leaf disks exposed to light under anaerobic conditions. In the dark, the synthesis of starch and sucrose from glucose was entirely suppressed by anaerobic conditions. It was concluded, therefore, that light could replace the oxygen requirement for synthesis in the tobacco leaf by inducing an anaerobic phosphorylation of glucose. Using glucose-1-^{14}C or glucose-6-^{14}C, it was shown that the distribution of ^{14}C in the sucrose and starch, which was synthesized anaerobically when the system was exposed to light, could be attributed predominantly to polymerization of hexose or triose units.

The synthesis and dissolution of starch has been studied by Porter, Martin & Bird (7a) in experiments in which detached starch-depleted tobacco leaves were allowed to assimilate equal amounts of $^{14}CO_2$ and $^{12}CO_2$ in succession, and vice versa, and the distribution of radioactivity in the starch, sugars, and CO_2 evolved was determined after assimilation and after disks cut from the leaves were kept in darkness for varying time periods. The results were discussed in relation to the hypothesis that starch grains are built up in layers and in relation to the currently accepted scheme for the synthesis and degradation of starch in green tissue by way of the phosphate esters of glucose and fructose. A starch like polysaccharide was formed during assimilation, which persisted in the dark.

Cellulose.—Wolfrom, Webber & Shafizadeh (8), by use of D-glucose-2-^{14}C and a new method for the determination of the label distribution within the anhydro-D-glucose units of cellulose-^{14}C, have confirmed previous conclusions that part of the glucose incorporated into cotton cellulose was first broken down and then resynthesized through the Embden-Meyerhof pathway.

Glycogen.—The concept that a mechanism for glycogen synthesis, which is not dependent on phosphorylase activity, is present in animal tissues now appears to be generally accepted. The important finding of Leloir & Cardini (9) that glycogen is synthesized from uridine diphospho-glucose by a liver enzyme has stimulated other authors to study this pathway of glycogen synthesis in muscle. Villar-Palasi & Larner (10), in a preliminary study, have described experimental conditions under which two enzymic reactions can be demonstrated in homogenates of rat skeletal and diaphragm muscle; together, these reactions constitute a sequence for the formation of glycogen from glucose-1-phosphate.

$$\text{Uridine triphosphate} + \text{glucose-1-phosphate} \rightleftharpoons \text{uridine diphospho-glucose} + \text{pyrophosphate} \quad 1.$$

$$\text{Uridine diphospho-glucose} + \text{glycogen} \rightleftharpoons (\text{glycogen} + 1) + \text{UDP} \quad 2.$$

Reaction 1 is catalyzed by the enzyme uridine diphospho-glucose pyrophosphorylase [first purified and studied in detail by Munch-Petersen (11) and recently studied in rat liver by Reid (12)]. From the equilibrium values of the two reactions, the authors concluded that, under physiological conditions, such a system would appear to greatly favor glycogen synthesis rather than breakdown. Robbins, Traut & Lipmann (13), working with homogenates of pigeon breast muscle, have described a system in which these homogenates incorporated glucose into glycogen. The incorporation was dependent on the presence of coenzyme-A in the system or of a muscle *kochsaft.* In order to determine whether phosphorylase activity was involved in this reaction, crystalline phosphorylase-*b* and a phosphorylase kinase were added; this led to an appreciable increase in phosphorylase-*a* concentration, which completely inhibited the incorporation of glucose into glycogen. ^{14}C-Labelled uridine diphospho-glucose was found to be incorporated in rat skeletal muscle homogenates much more rapidly than glucose or glucose-6-phosphate. Fractionation of the homogenates by differential centrifugation showed both the glucose and the uridine diphospho-glucose incorporation into glycogen in the particulate fraction with microsomal sedimentation characteristics. Leloir and his colleagues (14) have continued their studies on the biosynthesis of glycogen from uridine diphospho-glucose by use of a partially purified enzyme preparation from rat muscle. From a quantitative study of the rate of glucose transfer from uridine diphospho-glucose in this system and of the rate of glycogen synthesis, the authors concluded that the activity of the enzyme in these tissues is quite sufficient to account for all the glycogen synthesis. Furthermore, the authors have shown that, in the radioactive glycogen formed from ^{14}C-uridine diphospho-glucose, the glucose residues became attached by α-(1$\rightarrow$4) linkages, which is the type of linkage hydrolyzed by phosphorylase. The authors found that the reaction, which requires a polysaccharide as primer, was strongly activated by hexose-6-phosphates; the mechanism of this activation was not understood. The system synthesizing glycogen from uridine diphospho-glucose is present in various organs of the rat; the highest concentration was found in muscle, heart, and liver and a

small amount in brain, spleen, kidney, and lung. Hauk & Brown (15) have also reported a method for preparing, from rabbit skeletal muscle, a highly purified enzyme preparation that catalyzed glycogen synthesis from uridine diphospho-glucose, which has been shown to have an activity distinct from that of phosphorylase. The structure of the polysaccharide chain formed by this uridine diphospho-glucose-glycogen transferase enzyme was similar to that formed by phosphorylase.

Chen (16) has investigated the influence of a variation in the rate of oxygen flow on the synthesis from glucose-1-^{14}C of the polysaccharides mannan and glycogen and of the disaccharide trehalose in actively growing yeast. The results showed that there was a direct polymerization of hexose units in the synthesis of the polysaccharides independent of the rate of oxygen flow. The condensation of triose units via the Embden-Meyerhof pathway was insignificant.

Neptune & Foreman (17) have studied glycogen disappearance and lactate formation in rat diaphragm after 4 hr. incubation *in vitro*. They concluded that the glycogen that disappeared and was not accounted for as lactate would support only 10 per cent of the oxygen consumption of this tissue. This finding is consistent with earlier work showing that fatty acids are the major endogenous substrate for the support of respiration of excised rat diaphragm.

Mucopolysaccharides.—Pogell (18) has published some preliminary studies on the biosynthesis of mucopolysaccharides of bovine cornea from ^{14}C-glucose. He demonstrated a considerable formation of ^{14}C-hexosamine from ^{14}C-glucose, and the effect was stimulated by glutamine. The author suggested that glutamine reacted with glucose-6-phosphate to produce an increased glucosamine-6-phosphate synthesis. ^{14}C was shown to be present in both the glucosamine and galactosamine moieties of the polysaccharides. Spiro (19) has studied the rate of glucosamine turnover *in vivo* by injecting ^{14}C-glucose into rats and determining the specific activity time curves of the protein-bound glucosamine in various tissues. His results showed that liver is the principal site of synthesis of glucosamine and that a rapid interchange of glucosamine between liver and serum occurs. The rate of turnover of protein-bound glucosamine from glucose in the kidney, lung, testes, and spleen was well below that of liver.

Rodén & Dorfman (20) have studied the biosynthesis of some of the mucopolysaccharides of mammalian skin in relation to the uronic acid moiety of the molecule. They found that when glucose-6-^{14}C was administered to rats, the subsequent distribution of ^{14}C into the L-iduronic acid component of chondroitin sulphuric acid (β-heparin) and the D-glucuronic acid of skin hyaluronic acid showed that these substances were derived from the glucose molecule without cleavage of the latter. In both cases, the ^{14}C was equally distributed in the hexosamine moiety of the polysaccharide and in the carbon 6 of the uronic acid. Korn (21) has demonstrated the incorporation of ^{14}C-glucose and ^{35}S-sulphate into heparin in slices of mouse mast tumor cells.

He has shown (22) that, in the soluble fraction of these tumor cells, the incorporation of sulphate required the presence of ATP and that phosphoadenosylphosphosulphate was a probable intermediate in the transfer of inorganic sulphate to heparin. Dorfman, Markovitz & Cifonelli (23) have reviewed recent work on the biosynthesis of hyaluronic acid; much of this work has been carried out in their own laboratories during recent years. Bollet, Goodwin & Brown (24) have studied some of the enzymic steps involved in the synthesis of mucopolysaccharides in extracts of connective tissue of guinea pigs and of human synovial tissue. The finding in connective tissue of the enzymes (hexokinase, phosphoglucomutase, uridine diphosphoglucosepyrophosphorylase and -dehydrogenase) necessary to synthesize uridine diphosphate glucuronic acid from glucose suggested that the former is an intermediate in the synthesis of the glucuronic moiety of mucopolysaccharides. Glucuronosyl transferase, β-glucuronidase, and cytochrome-*c* reductase were also shown to be present in these preparations. Feingold, Neufeld & Hassid (25) have demonstrated the presence in mung-bean extracts, of a pyrophosphorylase capable of catalyzing the reversible formation of the two uronic acid nucleotides from uridine triphosphate and D-glucuronic acid-1-phosphate and D-galacturonic acid-1-phosphate.

uridine triphosphate + D-glucuronic acid-1-phosphate
$\rightleftarrows$ uridine diphosphate glucuronic acid-1-phosphate + pyrophosphate 3.

uridine triphosphate + D-galacturonic acid-1-phosphate
$\rightleftarrows$ UDP-galacturonic acid-1-phosphate + pyrophosphate 4.

It has not yet been established whether the pyrophosphorylase acting in these two reactions is the same enzyme or whether two enzymes are involved. In a more recent paper, Neufeld, Feingold & Hassid (26) have shown that mung-bean seedlings also contain an enzymic system capable of phosphorylating D-glucuronic acid in the presence of ATP, resulting in the formation of α-D-glucuronic acid-1-phosphate.

Glucans.—Feingold, Neufeld & Hassid (27) have shown that there is an enzyme in *Phaseolus aureus* (mung-bean) seedlings that transfers glucose units from the sugar nucleotide, uridine diphospho-glucose, to form a polysaccharide with β-(1$\rightarrow$3) glycosidic linkages shown to be identical with naturally occurring glucans. A partial purification of the enzyme was described. Since the D-glucose in uridine diphospho-glucose is α-glucosidically bound, the authors pointed out that there must be an inversion of configuration. Feingold *et al.* have demonstrated the presence of this enzyme system and of a laminarase enzyme that hydrolyzes glucans in seedlings of a number of higher plants.

Pectins.—Deuel & Stutz (28) have reviewed much of the recent work on pectic substances, including studies on the pectinesterases, the polygalacturonases, and the biosynthesis of these compounds. The action of yeast endo-polygalacturonase has been studied by Patel & Phaff (29).

Disaccharides.—The disaccharide trehalose is known to be an important carbohydrate storage material in insects. Candy & Kilby (30) have shown that, in locusts, the biosynthesis of trehalose from glucose occurs mainly in the fat body. The addition of ATP and uridine diphospho-glucose to a centrifuged, dialyzed extract of this tissue increased very considerably the synthesis of trehalose; from this, it was concluded that the biosynthesis of the disaccharide occurs via a phosphorylated intermediate. The authors suggested that the pathway of synthesis of trehalosephosphate in the locust is probably similar to that described by Cabib & Leloir (31) in yeasts:

$$\text{glucose-6-phosphate} + \text{uridine diphosphoglucose} \rightleftharpoons \text{UDP} + \text{trehalosephosphate} \quad 5.$$

Ayers (32) has shown that dried cell and cell-free enzyme preparations from *Ruminococcus flavefaciens* showed an uptake of inorganic phosphate in the presence of cellobiose with the accumulation of a mixture of phosphorylated hexoses. The data suggest that a cellobiose phosphorylase, which cleaves cellobiose to glucose-1-phosphate and glucose, is present. In the intact cell, glucose was not metabolized by this organism, and the author suggested that this may be caused by the absence of a specific permease for glucose, whereas such a permease enzyme may be present for cellobiose.

The presence of 2-0-β-D-glucopyranosyl-D-glucose (sophorose) and 6-0-β-D-glucopyranosyl-D-glucose (gentiobiose) have been detected by Khan, Pellegrino & Walker (33) in cultures of various strains of *Acetobacter* growing in defined media with glucose as carbon source. Walker, Pellegrino & Kahn (34) have shown these to be the two principal oligosaccharides formed in four strains of *Acetobacter.*

Malpress (35) has presented a detailed review on the present state of knowledge concerning lactose synthesis with a discussion of the enzyme systems involved and an extensive bibliography. Wood *et al.* (36), in one of a series of studies on lactose synthesis, have carried out a quantitative investigation on the constituents of milk after injection of DL-glycerol-1-^{14}C unilaterally into the pudic artery of a cow; the injected and non-injected sides of the udder were investigated separately, and the distribution of ^{14}C in the glucose and galactose moieties was determined. From their results Wood *et al.* concluded that the blood glucose is the precursor of the glucose moiety of lactose. The distribution of ^{14}C in the galactose moiety suggested the participation of the pentose cycle. This interpretation was confirmed in a second paper (37) by using the same technique and by injecting glucose-2-^{14}C and glucose-6-^{14}C and determining the ^{14}C distribution in lactose.

Transfer reactions in the synthesis of glycosidic bonds.—Hassid, Neufeld & Feingold (38) have reviewed much of the recent work on transglycosylation reactions involving the sugar nucleotides. The major pathway of glucuronide synthesis in liver is the enzymic transfer of glucuronic acid from uridine diphosphate glucuronic acid to an acceptor, which can be one of many glycoside-forming compounds [Storey & Dutton (39)]. Dutton & Stevenson (40) have now demonstrated this reaction in broken cell preparations of

kidney cortex and gastric mucosa, by use of *o*-aminophenol (-)-menthol and *o*-aminobenzoic acid as acceptors. The enzyme UDP-transglucuronylase of kidney was shown to resemble that of the liver in its optimum pH and in its confinement to the microsomes. The authors suggested that this pathway of glucuronide synthesis operates in the intact kidney and gastrointestinal tract since uridine diphosphate glucuronic acid can be isolated from these tissues. The nucleotide can be formed in these tissues from UDP, glucose, and DPN by the same mechanism as in liver. Dutton (41) has shown that there is a very low level of *o*-aminophenyl-glucuronide synthesis in foetal liver and kidney of guinea pig and man and in neonatal mouse liver because of the low levels of uridine diphosphate glucuronic acid and of the enzyme UDP-transglucuronylase. Both in the guinea pig and the mouse, normal glucuronide synthesis was rapidly assumed after birth.

Stetten (42) has obtained a transglucosylase activity in an alcohol precipitated powder from the supernatant solution of rat liver homogenates capable of transferring glucosyl residues from maltose, maltotriose, or glycogen to glucose, maltose or higher homologues, and water. All the reactions, with the exception of the transfer of the glycosyl residue to water, were shown to be reversible.

It was first shown by Hehre (43) that cell-free extracts from a culture of *Leuconostoc mesenteroides* were able to catalyze the polymerization of sucrose to dextrins. Neely (44) has studied the enzyme dextransucrase responsible for this reaction. He refers to previous work on the specificity of the enzyme, in which it was shown that sucrose is the specific donor for this reaction but that a number of sugars and sugar derivatives could act as acceptors. In the present work, Neely has shown that raffinose could act as an acceptor for dextransucrase to form a tetrasaccharide with a branched structure: O-α-D-galactopyranosyl-(1→6)-[(O-α-D-glucopyranosyl-(1→2)]-O-α-D-glucopyranosyl (1→2) β-D-fructofuranoside. Kinetic studies carried out by Neely (45) have shown that the enzyme dextransucrase showed a bifunctional catalytic site and the dissociation constants corresponded to a carboxyl and an imidazole ring. Neely (46) has produced further evidence that imidazole is implicated as part of the active center of dextransucrase by applying a photooxidation technique for destroying the imidazole portion of histidine in proteins.

Two papers of Bailey (47, 48) on the transglucosidase activity from sucrose of rumen strains of *Streptococcus bovis* have been published. The polysaccharides formed were demonstrated to be unbranched dextrans containing an α-(1→6)-glucosidic linkage; isomaltose was the sole disaccharide formed on partial acid hydrolysis, and detailed evidence was given to show that the dextrans produced were unbranched polysaccharides. The isolation and properties of the transglucosidase enzyme were described. Invertases from a number of plant tissues have been shown to synthesize oligosaccharides by transglycosidation. Henderson, Morton & Rawlinson (49) have found, in agreement with earlier workers, that starch is converted into glu-

cose, fructose, and sucrose during ripening of the banana. In addition, they have purified a trisaccharide from the ripening banana and have provisionally identified it as β-fructosyl sucrose with the structure O-β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1→2) β-D-fructofuranoside. Banana invertase was shown to catalyze the formation of oligosaccharides *in vitro* by a transfructosylation from sucrose to other sucrose molecules acting as acceptors; this probably also occurs *in vivo*.

Feingold, Neufeld & Hassid (50) have provided the first experimental evidence for an enzymatic transfer of pentosyl units. They found enzymatic activity in an asparagus extract which catalyzed the transfer of xylosyl units from UDP-D-xylose to xylosyl oligosaccharides (with a β-(1→4) linkage and a degree of polymerization of two to five units), which resulted in 1,4 linked oligosaccharide containing one more xylosyl unit than the acceptor. The question was discussed as to whether this enzyme could be involved in the synthesis of more complex polysaccharides. It was suggested that xylosides of plants, such as digitonin, ruberythric acid, and tomatin, may be synthesized by similar enzymes. Anderson & Manners (51) have shown that extracts of ungerminated barley contain enzymes which transfer α-glucosyl radicals from cellobiose to glucose, glucose disaccharides, and various pentoses. The oligosaccharides formed were isolated and characterized; they were gentiobiose and cellotriose (glucosyl-cellobiose). The enzymes were unable to synthesize oligosaccharides from glucose. The acceptor specificity of different cellobioses from various biological sources was discussed. Cardini & Yamaha (52) have shown that the plant glycoside gentiobiosyl-hydroquinone was synthesized by two transfer reactions when wheat-germ extracts were incubated with uridine diphosphoglucose. The first reaction was reported previously by Cardini & Leloir (53) and involves the formation of *p*-diphenol-β-glucoside (arbutin); the second reaction involved the glycosylation of glucose residues of arbutin at the 6 position to give gentiobiosyl hydroquinone. It has not yet been established whether one or two enzymes are involved in these reactions.

Hydrolytic enzymes.—Sutra (54) has published a general discussion on the properties of amylases. McGeachin & Reynolds (55) have shown that antibodies can be formed by mammalian amylases and have demonstrated differences in mammalian amylases from various tissues by the enzyme's inhibitory action of specific antisera. A study of the substrate specificity of rumen cellulolytic enzymes has been made by Festenstein (56). Pazur & Ando (57) have described the action and properties of an amyloglucosidase enzyme prepared from *Aspergillus niger* in a highly purified state, which converted starch, amylose, amylopectin, and amylodextrin to glucose in yields approximating complete conversion. Experiments carried out with maltooligosaccharides labelled in position 1 with ^{14}C as substrates demonstrated that the action of the amyloglucosidases on linear glucose polymers proceeded from the non-reducing end of the molecule. The hydrolytic enzymes glucanases have been shown by Reese & Mandels (58) to be present in different fungi as

constitutive enzymes, whereas, in most cases, other extracellular enzymes of fungi responsible for the hydrolysis of glycosidic bonds in different polysaccharides, such as amylases, cellulases, and chitinases, are adaptive enzymes.

Bailey & Clarke (59) have studied the properties of an extracellular bacterial dextranase secreted by two bovine rumen strains and one human strain of *Lactobacillus bifidus*. Moulds are the commonest source of extracellular dextranases, and it is of interest that the properties of the bacterial dextranases differ from those of the mould enzymes. Thus, the products formed by the action of bacterial dextranases on dextran were a mixture of isomaltotriose, tetraose, pentaose, and higher oligosaccharides; glucose and isomaltose, the main products of the action of other dextranases, were not formed. Furthermore, the bacterial dextranases hydrolysed dextrans with a minimum chain length of seven glucose units, whereas mould dextranases can hydrolyse chains from two to four glucose units in length. Phillips (60) has described a method of purification of maltase from a brewing strain of yeast, *Saccharomyces cerevisiae*, and has made a detailed study of the specificity of this enzyme.

Gottschalk (61) has reviewed the properties and mode of action of the enzyme neuraminidase, a glycosidase, which splits off the terminal sialic acid from the non-reducing end of disaccharides, oligosaccharides, and polysaccharides. The author discussed the evidence for the presence of this enzyme in influenza virus and *Vibrio cholerae* and for mucoprotein as a substrate of this enzyme.

Hexoses and Related Compounds

Metabolism.—In one of a series of studies, by Hastings and his colleagues, on carbohydrate metabolism in rat liver slices, alterations in the hexose metabolism associated with diets containing high concentrations of glucose or galactose have been reported (62). Using ^{14}C-labelled substrates, the authors showed that, following a high glucose diet, the conversion of glucose to CO_2, glycogen, and fatty acids was increased, whereas, following a high galactose diet, the conversion of glucose to glycogen and fatty acids was decreased and the conversion to CO_2 was unchanged. The authors suggested that these alterations are attributable to the amount of glucose-6-phosphate available to the liver for metabolism; thus, in animals maintained on a high glucose diet, there was an increase in the glucose phosphorylated in the liver, whereas, in animals maintained on a high galactose diet, glucose phosphorylation was decreased. The authors discussed the quantitative differences in liver carbohydrate metabolism when glucose, fructose, or galactose is provided as a substrate and the importance of the previous diet in any quantitative study of liver-carbohydrate metabolism.

Fitch, Hill & Chaikoff (63) have carried out a quantitative study on the glycolytic enzyme activities in the normal rat liver after the feeding of high concentrations of fructose in order to study the mechanism involved in the impaired capacity of the liver to utilize glucose after fructose feeding. There

was no evidence for a decline in activity of phosphatase, mutase, isomerase, glucose-6-phosphate dehydrogenase, or 6-phosphogluconic acid dehydrogenase after fructose feeding. The authors concluded that this finding is consistent with their earlier views that glucose utilization is blocked after fructose feeding at its initial phosphorylation. This may be attributable to a defect in the glucokinase system or in the mechanism responsible for bringing glucose to the site of glucokinase action in the cell.

Ashmore, Weber & Landau (64), utilizing glucose-1-^{14}C and glucose-6-^{14}C, have studied glucose metabolism in normal rat liver and in transplanted Novikoff hepatoma slices. They concluded from their results that of the four pathways of glucose-6-phosphate utilization known to occur in the normal liver, in the hepatoma, there was a complete absence of one pathway, glucose release, and a nearly complete abolition of another, glycogen synthesis; yet, the hexose-monophosphate shunt was present and lactic acid production was considerably increased. The authors maintained that these results agree essentially with data previously obtained by enzymatic studies on glucose-6-phosphate utilization in neoplastic liver.

The problem of the influence of hyperglycemia on hepatic-glucose output in the dog has been studied with ^{14}C-labelled glucose by Steele & Marks (65) and Steele, Bishop & Levine (66). On the basis of their work, this group of investigators do not support the concept (which has been frequently advanced) that hyperglycemia causes an inhibition of glucose output by the liver. Baker *et al.* (67) have measured the decline in specific activity of intravenously injected ^{14}C-glucose in rat and, by a mathematical analysis of their results, have calculated that the glucose space in the rat is approximately 20 per cent of the body weight and that the turnover time of plasma glucose is approximately 2 to 3 min.

The fate of generally labelled ^{14}C glucose in the isolated rat pituitary gland has been studied by Beloff-Chain and co-workers (68) using a quantitative paper chromatographic technique (69) and an automatic scanning device (70). All the glucose metabolized by the tissue after 3 hr. incubation was accounted for as alanine, aspartic acid, glutamic acid, proline, lactic acid, CO_2, small amounts of glycogen, oligosaccharides and glycerol, protein-bound aspartic acid, glutamic acid, alanine, and proline. A qualitative and quantitative comparison of glucose metabolism in the pituitary gland and in brain slices was presented. Insulin was shown to have no stimulating effect on glucose metabolism in the pituitary gland, and no abnormality was observed in glands from alloxan diabetic rats. In pituitary gland and brain slices from vitamin-B_6 deficient rats, there was a considerable diminution of alanine formation from glucose, whereas all the other amino acids were formed normally.

The incorporation of uniformly labelled ^{14}C glucose and of galactose-1-^{14}C into the neutral glycolipid fraction of rat brain tissue has been studied by Burton, Sodd & Brady (71) at various times after birth. Experiments *in vitro* showed that the microsomal fraction of young rat brain contains the enzymes

necessary for the incorporation of glucose and galactose into neutral glycolipids. The authors suggested the following pathway for glucose:

Glucose + ATP $\rightleftharpoons$ glucose-6-phosphate 6.

Glucose-6-phosphate $\rightleftharpoons$ glucose-1-phosphate 7.

Glucose-1-phosphate + uridine triphosphate $\rightleftharpoons$ uridine diphospho-glucose + pyrophosphate 8.

Uridine diphospho-glucose $\rightleftharpoons$ UDP-galactose 9.

UDP-galactose + acceptor $\rightleftharpoons$ neutral glycolipid + UDP 10.

and for galactose:

Galactose + ATP $\rightleftharpoons$ galactose-1-phosphate + ADP 11.

Galactose-1-phosphate + uridine diphospho-glucose $\rightleftharpoons$ UDP-galactose + glucose-1-phosphate 12.

UDP-galactose + acceptor $\rightleftharpoons$ neutral glycolipid + UDP 13.

An excellent review on the metabolism of UDP-galactose and the enzyme reactions in which it is involved has been recently published by Kalckar (72).

Ehrlich & Segel (73) have studied the carbon balance in *Bacillus megatherium* growing in a glucose mineral medium. Under conditions of forced aeration, it was shown that the end products formed were CO_2, lactate, acetate, pyruvate, glycerol, acetoin, and 2,3-butanediol. Mansour (74) has studied carbohydrate metabolism in the liver fluke, *Fasciola hepatica*, a metazoan parasite which lives in the bileduct under predominantly anaerobic conditions. The parasites were shown to metabolize glucose rapidly under anaerobic conditions, and the production of propionic and acetic acids accounted for almost all the carbohydrate utilized; very little lactic acid was formed. Glycogen was utilized at a high rate when the flukes were cultured in a medium without glucose, but, when glucose was present, glycogen was conserved.

Two papers on the effect of substitution of glucose by other carbohydrates in cell culture media have been published. Eagle and co-authors (75) have studied the utilization of D-fructose, D-galactose, D-mannose, D-ribose, and trehalose by normal and malignant human cells. They found that mannose and fructose could regularly substitute for glucose, whereas the growth response to galactose was variable and was increased by the addition of pyruvate. A number of strains were able to utilize D-ribose within a narrow concentration range. Large differences in lactic acid production were found with different substrates. Eagle *et al.* found that the carbohydrate utilization (which was proportional to the substrate concentration of the medium) did not correspond to cell growth. From their results, they suggested that the amount of carbohydrate metabolized, the efficiency of its utilization, and the extent of aerobic glycolysis were interrelated and depended on the rate at which substrate becomes available to the cells as glucose-6-phosphate or fructose-6-phosphate more than on the cell type or on the concentration re-

quired for optimum growth. The pattern of carbohydrate utilization by normal and malignant cells was similar. Bailey, Gey & Gey (76) have studied the effect of replacing glucose by a large number of other sugars and related compounds on the growth of a strain of mouse lymphoblasts. D-Mannose and D-glucosamine were shown to support growth in a manner comparable to D-glucose, D-fructose, D-galactose, D-sorbitol, and glycerol; lactic acid supported growth less efficiently and L-sorbose, D-mannose, and D-mannitol still less, although there was still a prolonged survival of cells as compared with that observed in a sugar-free medium. The authors presented a schematic representation of the probable mode of entry into the glycolytic pathway of the compounds studied. A large number of other substances tested by the authors were shown to be inactive.

Vittorio, Spence & Johnston (77) have studied the metabolism of glucose and fructose in rat liver slices after exposure of the animals to x-rays. They found that liver slices taken 4 hr. after irradiation of the animals incorporated more ^{14}C from glucose and fructose into CO_2, glycogen, and an ethanolic extract of the tissue than liver slices from normal rats; after 24 hr. similar results were obtained, except that glucose conversion to CO_2 was lower after irradiation. The effect of whole body x-irradiation on increasing glucose oxidation has also been observed in rat small intestine mucosa *in vitro* by Kay & Entenman (78). The effect was observed within 3 hr. after the exposure to x-rays, and from 3 to 48 hr. after the glucose oxidation remained constant; however, on the third postirradiation day, the rate increased and the increase persisted during the fourth day. Using ^{14}C-labelled intermediates, the authors concluded that the increase in the oxidation of glucose to CO_2 and lactic acid after x-irradiation was attributable almost entirely to increased citric acid cycle activity. The Embden-Meyerhof pathway of glucose oxidation did not appear to be affected.

Biosynthesis.—Hagerman, Roux & Villee (79) have studied the mechanism of fructose production by human placenta *in vitro*. Hers (80) demonstrated the presence, in sheep seminal vesicles of two enzymes, an aldose reductase and a ketose reductase, which catalyzed the following reactions respectively:

$$\text{Glucose} + \text{TPNH} + \text{H}^+ \rightleftarrows \text{sorbitol} + \text{TPN} \qquad 14.$$

$$\text{Sorbitol} + \text{DPN} \rightleftarrows \text{fructose} + \text{DPNH} + \text{H}^+ \qquad 15.$$

These reactions provide a mechanism of fructose synthesis which does not involve phosphorylated intermediates. Hagerman *et al.* (79) have shown that whole homogenates of human placenta produced fructose when incubated with fructose-6-phosphate or sorbitol and DPN. The placenta was shown to contain ketose reductase but not an aldose reductase or significant amounts of sorbitol. The authors concluded that fructose is formed in the placenta via the intermediate formation of the phosphorylated sugars and the action of a non-specific phosphatase. King & Mann (81) have partially purified, from ram spermatozoa, the enzyme sorbitol dehydrogenase, which catalyzes

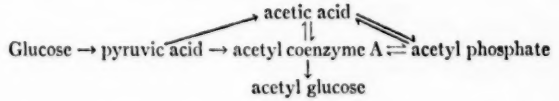
the oxido-reduction of sorbitol to fructose in the presence of DPN. The authors found that the enzyme from spermatozoa is similar in its mode of action to the enzymes previously obtained from rat liver and male accessory organs. The enzyme D-sorbitol-6-phosphate dehydrogenase, which catalyzes the interconversion of D-sorbitol-6-phosphate and D-fructose-6-phosphate, has been isolated, partially purified, and its properties studied by Shockley & Pride (82).

Yamada *et al.* (83) have studied the biosynthesis of mannitol in the fungus *Piricularia oryzae*. They have demonstrated the presence of two enzymes, a fructose-6-phosphate-DPN dependent reductase, which catalyzes the formation of mannitol-1-phosphate from fructose-6-phosphate, and a mannitol-1-phosphatase. Their results, therefore, indicate that mannitol is formed by the following mechanism:

$$\text{Fructose-6-phosphate} \rightarrow \text{mannitol-1-phosphate} \rightarrow \text{mannitol} \qquad 16.$$

The rapid turnover of sorbitol in plum leaves has been demonstrated by Anderson, Andrews & Hough (84) in experiments in which leaves were allowed to photosynthesize in an atmosphere of $^{14}CO_2$, and the ^{14}C of sorbitol was measured at various time intervals.

It was first shown by Duff, Webley & Farmer (85) that 6-O-acetyl-D-glucopyranose is a major metabolite of a cobalamin-producing strain of *Bacillus megatherium*. Duff & Webley (86) have studied the mechanism of formation of acetyl-glucose in this strain grown on a glucose or sucrose medium. Pyruvate was shown to be a key intermediate in the biosynthesis of the ester, and acetic acid was shown to be produced at the same time as acetyl glucose. Both acetic acid and pyruvate were shown to stimulate the reaction. On the basis of these data and the finding of a phosphotransacetylase enzyme and an acetyl coenzyme A kinase in cell free extracts of the organism, the authors suggested the following possible pathways which might operate for the synthesis of acetyl glucose:



17.

The final stage would require a specific condensing enzyme. The authors emphasized that the reactions leading to the formation of acetic acid or acetyl coenzyme A from pyruvic acid and the interconversion of acetic acid and acetyl phosphate require further investigation. A specific 6-O-acetyl-D-glucopyranose deacetylase was also present in the cell-free extracts of the organisms, and the authors suggested that this enzyme could also be involved in the synthesis of the ester.

Pentoses

Metabolism.—Jonsen, Laland & Strand (87) have demonstrated the presence of a deoxyribokinase in cell-free extracts from *Escherichia coli* mutants

adapted to deoxyribose, which catalyzes the phosphorylation of 2-deoxyribose in the presence of ATP and Mg^{++} to form 2-deoxyribose-5-phosphate. This same enzyme has been isolated and partially purified from *Lactobacillus plantarum* (88) grown on 2-deoxyribose.

The occurrence of D-arabinose-5-phosphate as an intermediate in the metabolism of pentoses by *Propionibacterium pentosaceum* has been described by Volk (89). He showed with enzyme extracts from these microorganisms that D-arabinose-5-phosphate could be formed from either L-arabinose or D-ribulose plus ATP or from D-ribose-5-phosphate. The author proposed the following scheme, involving two epimerisations, for the formation of D-arabinose-5-phosphate from L-arabinose and ATP:

$$\text{L-Arabinose} \rightleftharpoons \text{L-ribulose} \rightarrow \text{ribulose-5-phosphate} \rightleftharpoons \text{D-xylulose-5-phosphate} \rightleftharpoons \text{D-ribulose-5-phosphate} \rightleftharpoons \text{D-arabinose-5-phosphate} \quad 18.$$

Jensen has reported (90) that different strains of *Azotobacter*, grown on arabinose or xylose as the sole carbon source, formed an unidentified acid. This observation may be related to the finding of Weissbach & Hurwitz (221) who isolated and identified 2-keto-3-deoxyheptonic acid in crude cell-free extracts of *E. coli* incubated with ribose-5-phosphate (see page 320–21); this acid may represent an important intermediary in the metabolism of the pentose. Another 2-keto-3-deoxy sugar, L-2-keto-4,5 dihydroxy valeric acid has been shown by Weimberg (91) to be an intermediate of L-arabinose oxidation by *Pseudomonas saccharophila*. The present state of knowledge of the overall reaction sequence of L-arabinose metabolism in *P. saccharophila* has been presented by Weimberg in the following scheme:

$$\text{L-Arabinose} \rightarrow \text{L-arabono-}\gamma\text{-lactone} \rightarrow \text{L-arabonate} \rightarrow \text{L-2-keto-4,5-dihydroxy-valerate} \rightarrow \alpha\text{-ketoglutarate} \quad 19.$$

D-xylose was reduced to D-xylitol (92) in cell-free extracts of *Penicillium chrysogenum* in presence of TPNH. Xylulose could not be detected as an intermediate. D-Ribulose has been obtained by Fromm (93) from ribitol by the action of the DPN-dependent ribitol dehydrogenase, which has been isolated and purified from *Aerobacter aerogenes*. The enzyme which catalyzes the reaction

$$\text{Ribitol} + \text{DPN} \rightleftharpoons \text{D-ribulose} + \text{DPNH} + \text{H}^+ \quad 20.$$

appears to be specific for ribitol.

Biosynthesis.—It has been established that the synthesis of pentoses from hexoses can take place by three different pathways: (*a*) the oxidation of carbon 1 of the hexose and the subsequent loss of this carbon atom as CO_2, (*b*) a reaction between the hexose phosphate and triose phosphate molecules involving transketolases and transaldolases, and (*c*) the oxidative removal of carbon atom 6 of glucuronic acid. Hiatt & Lareau (94) have now clearly shown, by injecting glucose labelled with ^{14}C in carbon atoms 3 and 4, that, in the rat, the biosynthesis of ribose was almost entirely via pathway *b* and only a very small amount was formed through pathway *c*. In this connection,

it is of interest that Elzina & Engelhardt (95) have reported that, after aerobic incubation of glucose-1-^{14}C with ascite-cancer cells, the ribonucleic acid pentose exhibited twice as great a specific activity as after incubation with glucose-6-^{14}C. The author therefore suggested that the principal pathway of formation of RNA pentose in the tumor cells is the transketolase-transaldolase one, i.e., pathway *b*. Horecker, Domagk & Hiatt (96) have shown in regenerating liver, in ascites tumor cells, and in human carcinoma cells, that the pattern of labelling in deoxyribose formed from specifically labelled glucose was similar to that found in ribose. In addition, these authors have shown that, with glucose-1-^{14}C, a considerable amount more radioactivity was recovered in carbon 5 of deoxyribose than ribose. This indicates the possibility of another minor pathway involving the formation of triose phosphate as precursor of deoxyribose. Shreeve (97) has also suggested, from experiments in which glycine labelled with ^{14}C in carbon atoms 1 and 2 were injected into partially hepatectomized rats, that deoxyribose may be formed, at least in part, in regenerating liver by a route which does not involve ribose as an immediate precursor. Bagatell, Wright & Sable (98) have found that, in *Escherichia coli* grown on a synthetic medium with sodium acetate as sole carbon source and with glucose-6-^{14}C as tracer, the distribution of ^{14}C in the deoxyribose molecule was very similar to that in the ribose molecule; thus they have demonstrated that, in this organism also, the main pathway of formation of deoxyribose is reduction of ribose. In agreement with these findings are the results of Wacker, Kirschfeld & Träger (99) who showed that the ribose of ^{14}C-labelled nucleosides is transformed in *E. coli* and in *Lactobacillus leichmannii* into deoxyribose without a cleavage of the carbon skeleton.

Southard, Hayashi & Barkulis (100) have isolated L-rhamnose from the cell walls of group A streptococci grown in a medium containing glucose labelled with ^{14}C in carbon atoms 1 and 6. They found 90 per cent of the radioactivity from carbon 1 of glucose in the carbon 1 of the rhamnose molecule and 80 to 90 per cent of the activity of carbon 6 of glucose in carbon atoms 5 plus 6 of rhamnose; the results indicated clearly that the biosynthesis of rhamnose from glucose occurred without a cleavage of the hexose carbon skeleton.

Glycolysis

General considerations.—McIlwain (101) has shown that the increase in the aerobic glycolysis of the rat cerebral cortical tissue produced by thiol compounds was associated with a decrease in phosphocreatine. Traub & Ginzburg (102) have demonstrated that the inhibitory effect of fluoropyruvic acid on respiration in mitochondrial preparations with a high aerobic glycolysis was abolished in the presence of glucose, fructose, or mannose. The authors have tentatively suggested that this effect can be explained by a rapid reduction of the inhibitor to fluorolactate by means of the glycolytic cycle. Krimsky (103) found that, in a system composed either of glycolytic

enzymes or respiring rat liver mitochondria, it was possible to obtain the synthesis of phosphoenol pyruvic acid by reversal of pyruvate kinase; furthermore, in the mitochondrial system in the presence of appropriate enzymes, the formation of glycerophosphate and fructose-1-6-diphosphate from pyruvate was also demonstrated. Graymore, Tansley & Kerly (104) have shown that there was a large decrease in the anaerobic glycolytic activity of retina taken from rats with a hereditary visual degeneration as compared with the normal rat retina.

Occurrence of glycolytic systems.—Abood, Brunngraber & Taylor (105) have confirmed that the enzymes of the glycolytic pathway are present in rat brain mitochondria in contradistinction to the mitochondria of the heart, kidney, and liver, in which, as is known, the glycolytic system is practically absent. Bartlett (106) has isolated in good yields from erythrocytes intermediates of glycolysis by use of a chromatographic technique on ion exchange columns (107). Bernstein & Sweet (108) have provided data which suggest that most of the enzymes of glycolysis are present in the skin of young rats. Futterman & Kinoshita (109) have demonstrated the presence of the glycolytic pathway in homogenates of cattle retina. Partially purified extracts of pea-seeds have been shown (110) to possess a glycolytic system which transforms starch and hexoses to ethanol and CO_2. Syrett (111) has studied the fermentation of glucose by *Chlorella vulgaris* with specifically labelled ^{14}C-glucose. The distribution of ^{14}C in the acetic acid and lactic acid formed indicated that glucose was split by the Embden-Meyerhof glycolytic reactions into two C_3 fractions which were either converted to lactic acid or decarboxylated to a precursor of acetic acid. Data from the amount of ^{14}C-labelled carbon dioxide formed from specifically labelled glucose indicated that reactions other than those of the Embden-Meyerhof pathway were also involved.

Pasteur Effect.—It is generally accepted that tissues respond to anaerobic conditions by increasing the formation of lactic acid from glucose. Such an effect has been recently observed in rat diaphragm muscle (112), in brain mitochondria (113), and in tumour cells (114). It is, however, difficult to interpret these results particularly when lactic acid is determined by a chemical method. In fact, using ^{14}C glucose and a technique previously mentioned (69, 70), which enables the fate of the glucose to be followed quantitatively under different experimental conditions, Beloff-Chain and co-authors have shown that, in rat brain slices (115) and in the isolated rat diaphragm (116), the formation of lactic acid from glucose, under strictly anaerobic conditions, was reduced by about one-fifth in brain tissue and by about one-half in muscle as compared with the lactate produced under aerobic conditions. This result with diaphragm muscle is not in agreement with that reported by Randle & Smith (112) and the discrepancy may be attributed to differences in experimental technique. In this connection, an important observation (116) is that, if the muscle was preincubated in oxygen for only a few minutes and then incubated in nitrogen, there was a rapid and large production of

lactic acid from glucose. This effect was only obtained if glucose was in contact with the muscle during the aerobic phase; if, however, the labelled glucose was added only during the anaerobic phase to muscle which had been previously incubated aerobically, no increase in radioactive lactic acid formation was observed. The authors interpreted these results as an indication that some intermediate, or intermediates, must be formed, during the aerobic phase, that are essential for transformation of glucose to lactic acid and are rapidly transformed into lactic acid in the anaerobic phase.

In a recent review, Racker & Gatt (117) have discussed the interrelationship between glycolysis and oxidative pathways. No new ideas have been advanced to explain the Pasteur effect, partly because most of the work published during the period covered by this review was an extension of preliminary work previously published and discussed in detail by Holzer (118). Thus, further evidence has been given that the following phenomena are involved in the Pasteur effect: competition between the respiratory system and the glycolytic system for inorganic phosphate and ADP (119); the inefficient utilization of the ATP, which accumulated aerobically in the mitochondria, for the phosphorylation of glucose by the hexokinase reaction (114); and the rate-limiting role of triosephosphate dehydrogenase in glycolysis (113).

Hatch & Turner (120) have demonstrated that the aerobic inhibition of glycolysis in pea-seed extracts was caused by the oxidative inactivation of glyceraldehyde-3-phosphate dehydrogenase. The authors showed a reversal of the aerobic inhibition of glycolysis by cysteine and reduced glutathione and suggested that glyceraldehyde-3-phosphate dehydrogenase was inactivated through oxidation of essential —SH groups. Aisenberg (121) has provided further evidence that the Pasteur effect is caused by the inhibition of phosphohexokinase by measuring the concentration of hexose-diphosphate, glucose-6-phosphate, and fructose-6-phosphate in a system in which the Pasteur effect was obtained by adding a mitochondrial liver preparation to a glycolysing system. Birkenhäger (122) has shown that, in certain tumour cells in which glycolysis was little affected by oxygen, it was inhibited by ferrocyanide. Using specifically labelled ^{14}C-glucose, he showed that the inhibition of glycolysis was at the phosphoisomerase or phosphohexokinase level. Von Korff (123) has reported that, in a system containing the enzymes that catalyze the transformation of fructose diphosphate to phosphoglyceric acid, lactic dehydrogenase, and washed heart-muscle mitochondria, aerobic phosphorylation limited the glycolytic reactions by competition for ADP at the phosphoglycerokinase level.

The fact that tumour tissue, when incubated with glucose, manifests a decreased respiration (Crabtree effect) has been interpreted by Wu & Racker (114) as a competition between the two systems for inorganic phosphate and, under some conditions, for adenine nucleotides. Gatt & Racker (124) came to the same conclusion by working with a multienzyme system constituted from a glycolytic system and respiring rat liver mitochondria. Dewey &

Green (125), from investigations with Ehrlich ascites tumour cells, were in agreement with the concept that a decrease in intracellular pH is the cause of the Crabtree effect. Ibsen, Coe & McKee (126) interpreted the decrease in respiration by glycolysis in Ehrlich ascites carcinoma cells as a deficiency of ADP within the mitochondria. Abdel-Tawab, Broda & Kellner (127) have shown that, in monolayers of chick embryo mesenchyma tissue and of tumour tissue, the shift from respiration to aerobic glycolysis with an increasing supply of glucose did not influence the enzymes of the tricarboxylic acid cycle. Quastel & Bickis (128) have found that, in Ehrlich ascites carcinoma cells, sarcoma 37, Novikoff hepatoma, and a melanoma, the relationship between the rate of aerobic glycolysis [$Q_{CO_2\,(O_2)}$] and the difference in the rate of respiration determined in absence of glucose (Q_{O_2}) and in the presence of glucose [$Q_{O_2\,(G)}$] was constant and approximately equal to six. From these data and from the finding that the ratio of phosphorus to oxygen (P/O) was about three and that two molecules of ATP were formed via the glycolytic cycle for one molecule of glucose, the authors deduced the following relationship:

$$[Q_{O_2} - Q_{O_2(G)}] \cdot 2P/O = Q_{CO_2(O_2)} \qquad 21.$$

The relationship demonstrates that phosphorylation and respiration are closely coupled in intact tumour cells. This relationship is only valid if the concentration of ATP in the cells is the same both in the presence and absence of glucose. This means that glycolysis produced an energy equivalent to that lost by the decreased oxygen consumption. This hypothesis has also been confirmed by Ibsen, Coe & McKee (129) in Ehrlich ascites carcinoma cells. Creaser, De Leon & Sholefield (130), using ^{32}P, agreed with the hypothesis of Quastel & Bickis that the inhibition of respiration in Ehrlich ascites cells does not affect the distribution of radioactivity on the adenosine polyphosphates.

Glycolytic enzymes.—The properties of hexokinase from bakers' yeast have been studied in detail by Sols *et al.* (131). The maximum activity of the enzyme was found at pH 7.5, and the maximum stability was at pH 5.8. The properties were shown to be very similar to those of brain hexokinase, although some significant differences have been observed. In metabolic studies, 2-C-hydroxymethyl-glucose was shown to be a useful inhibitor of hexokinase, as it has more affinity for the enzyme than does N-acetyl glucosamine and, furthermore, has the advantage of not being a metabolite. Agosin & Aravena (132), in studies on the metabolism of *Echinococcus granulosus*, have demonstrated the presence of four specific hexokinases in cell-free preparations of hydatid cyst scolices, which, in the presence of ATP and Mg^{++}, specifically phosphorylated the carbon 6 of glucose, fructose, mannose, and glucosamine. A specific hexokinase that catalyzed only the phosphorylation of glucose has also been found in cell-free extracts of *Clostridium tetani* by Martinez & Rittenberg (133). McComb & Yushok (134) have partially purified and investigated the properties of hexokinase obtained from Krebs-2 ascites carci-

noma. It was shown to act on 11 different sugars and did not differ in its properties from the hexokinase isolated by Sols & Crane (134a) from brain.

Holldorf and co-authors (135) have purified 16 to 18 times the DPN dependent aldehyde dehydrogenase from cattle liver and have extended the work of Lamprecht & Heinz (136) with this purified preparation, thus confirming that the dehydrogenase that acts on glyceraldehyde is the same dehydrogenase enzyme as described by Racker (137) to act on acetaldehyde.

It is known that there are two types of phosphoglyceromutase enzymes, differing in their dependence on 2,3-diphosphoglyceric acid as coenzyme. Grisolia & Joyce (138) have examined the activity of phosphoglyceromutase in a large number of biological sources, including animal tissues, bacteria, yeasts, vegetables, and seeds, and have found that the enzyme is stimulated by 2,3-diphosphoglyceric acid in all the biological material studied, with the exception of preparations from seeds (138, 139). The authors suggested that, since many tissues synthesize more 2,3-diphosphoglyceric acid than is necessary for the activation of phosphoglyceric acid mutase, it is probable that this product has some other metabolic function.

Joyce & Grisolia (140) have partially purified the enzyme diphosphoglyceromutase from chicken breast muscle, and Krimsky (141) has provided further confirmation for the formation of 2,3-diphosphoglyceric acid from 1,3-diphosphoglyceric acid in rabbit skeletal muscle.

MacLeod & Wróblewski (142) have shown that lactic dehydrogenase is present in high concentration in normal human semen. Solomon (143) has demonstrated its presence in the chick embryo. Wieme (144), using a technique which combines electrophoresis on agar and a spectrophotometric enzyme test, has given further evidence for the "multiplicity" of the lactic acid dehydrogenase. The author showed that, in renal and cardiac tissue of rat and white mouse, there are five lactic dehydrogenases, whereas, in the plasma and the liver, there are three. Moore & Wortman (145) have found three forms of lactic dehydrogenase extractable from rabbit cornea by using an ion exchange method of separation. Tubbs & Greville (146) have purified a lactic dehydrogenase from rabbit kidney mitochondria and have studied its properties. Haugaard (147) also showed the presence of D-lactate dehydrogenase in enzyme preparations from *E. coli*. A lactic oxidase, which does not appear to be DPN linked, has been shown to be present in the protozoan *Tetrahymena pyriformis* (148); the pyruvate formed from lactate in presence of this enzyme corresponded stoichiometrically to the oxygen consumed, according to the reaction:

$$CH_3CHOH—COOH + \tfrac{1}{2}O_2 \rightarrow CH_3—CO—COOH + H_2O \qquad 22.$$

The Tricarboxylic Acid Cycle

General observations.—Vishwakarma & Lotspeich (149) have shown that, when some of the intermediates of the tricarboxylic acid cycle, such as citrate, α-ketoglutarate, and succinate, were administered to dogs by constant intra-

venous infusion, the renal tubules secreted other members of the cycle, such as malic acid. Furthermore, they found that, during the infusion of fumarate or malate, the capacity of the tubules to reabsorb malate was greatly increased, so that the tubules appeared to be capable of secreting or absorbing malate depending on the experimental conditions. On the basis of these results, the authors have suggested that the tricarboxylic acid cycle, generally considered as an oxidative mechanism for the supply of energy, can also function in the kidney as a mechanism necessary for synthesis, reabsorption, and secretion of its intermediates.

Evidence has been obtained for the operation of the cycle in apple tissue slices by Hatch *et al.* (150). DeLuca *et al.* (151) found that, when inorganic phosphate was added to kidney homogenates, it inhibited the oxidation of citrate and isocitrate but not of α-ketoglutarate, glutamate, fumarate, pyruvate, and succinate. The highest rate of citrate oxidation was obtained with kidney tissue followed, in decreasing order of intensity, by liver, brain, heart, and spleen tissue.

Oxidation of pyruvate and of α-ketoglutarate.—Reiss (152) has observed that the block in the utilization of pyruvate in rat heart sarcosomes by γ-(*p*-arsenophenyl)-*n*-butyrate could be eliminated by the addition of lipoic acid and that the reaction was stereospecific so that only the dextrorotatory isomer of lipoic acid was active in this particular system. Sanadi, Langley & Searls (153), in their studies on the α-ketoglutarate dehydrogenase system in hog heart, have also shown that the dextrorotatory lipoic acid was much more active than the laevo isomer. Furthermore, they have reported that the amide of lipoic acid was more active and saturated the enzyme at a lower concentration than free lipoic acid. In agreement with this finding is the result of Massey (154) with pig heart diaphorase; in these preparations, the author found that DL-lipoamide was about 350 times as active as lipoic acid. Koike & Reed (155) have purified an enzymic system obtained from *E. coli*, which catalyzed the oxidative decarboxylation of pyruvate, and, in this system, a flavine, tentatively identified as flavin-adenine-dinucleotide, is an essential component and is presumably associated with dehydrolipoic acid. Sanadi, Langley & White (156) have suggested that, in *E. coli*, the oxidative decarboxylation of α-ketoglutaric acid forms an active succinic compound as intermediate, probably succinyl-S-dithioloctanoate, which can then be transferred to coenzyme A.

Goldman (157) has separated, from *Mycobacterium tuberculosis*, the pyruvic acid dehydrogenase complex into two fractions: one contained the lipoic dehydrogenase which catalyzes the oxidation of the reduced form of lipoic acid; the other fraction contained, in addition to pyruvic dehydrogenase, a lipoic transacetylase which catalyzes the reversible transfer of an acetyl group from the acetyl-coenzyme A to reduced lipoic acid. Associated also with these two enzymes was a lipoic deacylase which catalyzes the hydrolysis of S-acetyl-lipoic acid to acetate and reduced lipoic acid. From the results obtained with the pyruvic acid dehydrogenase system, Goldman sug-

gested that the true coenzyme of this reaction is not lipoic acid, either in the free form or protein bound, but is probably lipoamide; this conclusion is in excellent agreement with the results discussed above and obtained with animal tissue.

Enzymes of the cycle.—Englard (158), using deuterium labelling, has shown that the substrate for the condensing enzyme (obtained from pig heart) in the synthesis of citrate from acetyl-coenzyme A and oxalacetate was the keto form of oxalacetate and not the enolic form.

Peters (159), working with kidney particles, found that fluorocitrate inhibited the soluble aconitase less than the aconitase present in mitochondria; furthermore, he has excluded the possibility that fluorocitrate reacts by blocking the formation of α-ketoglutaric acid (160). Ruffo, Romano & Adinolfi (161) have confirmed that oxalacetate and glyoxylate react together chemically to form a compound which inhibits the oxidation of citric acid; they showed that crude preparations of aconitase from rat liver or purified preparations from pig heart were inhibited when incubated in the presence of glyoxylate and oxalacetate. The chemical nature of this inhibitory compound has not been established, but the authors suggested that it may be a tricarboxylic acid with six carbon atoms. Purvis (162) has given further proof that both in rat liver and in rat heart mitochondria isocitric dehydrogenase is TPN specific.

It is known that α-ketoglutaric acid can be oxidized via a shunt of the tricarboxylic acid cycle; reacting with γ-amino-butyric acid, it forms glutamic acid and succinic semialdehyde which is then oxidized to succinic acid. Scott & Jakoby have now isolated from *Pseudomonas fluorescens* both the transamination enzyme (163) of this reaction and the oxidative enzyme (164); the reaction catalyzed by succinic semialdehyde dehydrogenase does not appear to be reversible.

$$\text{Succinic semialdehyde} + \text{TPN} + \text{H}_2\text{O} \rightarrow \text{succinate} + \text{TPNH} + \text{H}^+ \qquad 23.$$

Lara (165) has isolated and purified a succinic dehydrogenase from *Propionibacterium pentosaceum* and has shown that this enzyme is cytochrome-*b* linked, as is the case with succinic dehydrogenase, from other bacteria. Shull (166) has demonstrated the presence of a DPN dependent L-malic dehydrogenase in the microsome fraction of horse and rat liver, and Solomon (143) has established the presence of this enzyme in the chick embryo. Lorini & Moret (167) have found that, in *Aspergillus niger*, when the activity of oxalacetic decarboxylase increased, there was an increase in citric acid production.

Metabolism of the acids of the tricarboxylic acid cycle and related compounds.—Bartley *et al.* (168) have shown that the washed particles of rat liver under anaerobic conditions utilized about 50 per cent of the citrate utilized aerobically. The citrate metabolized anaerobically was accounted for approximately as 10 per cent malate and fumarate, 4 per cent α-ketoglutarate, 32 per cent glutamate, 34 per cent succinate, and 20 per cent lac-

tate, hydroxybutyrate, and carbohydrates. The authors found that the presence of ATP, DPN, and TPN increased the utilization of citrate, whereas dithionite, hydroxylamine, and ethylenediaminetetraacetate decreased its utilization and cyanide abolished it completely.

Vickery (169) has found that excised tobacco leaves utilized three times more malate in the presence of malonic acid than in its absence; succinate and citrate accumulated in the presence of malonate but the latter more slowly than the former. These results may be explained by the experiments of Shannon, Young & Dudley (170) who, by using malonic acid ^{14}C-labelled in carbon 2, have shown that, in plants, the C-2 of malonic acid was incorporated in large amounts into malic acid and to a smaller extent, varying in the different species studied, into citric acid, succinic acid, and fumaric acid. In addition, a considerable amount of radioactivity was incorporated into other acid constituents, which have not as yet been identified. These results indicate that the enzyme (or enzymes) capable of metabolizing malonic acid may be very widely distributed in the plant kingdom. Young & Shannon (171) found, in bush-bean leaves, that malonate-2-^{14}C was converted into ^{14}C-labelled acetate under anaerobic conditions. The conversion probably occurred via the intermediate formation of malonyl-coenzyme A.

Walker (172) has found that suspensions of *Lactobacillus brevis*, unlike homofermentative lactobacilli which transform pyruvate into acetoin, metabolized pyruvate to acetate and carbon dioxide by two distinct steps. Walker suggested that the first step may involve the dismutation of pyruvate to form lactate, acetate, and CO_2:

$$2\ \text{Pyruvate} \rightarrow \text{lactate} + \text{acetate} + CO_2 \qquad 24.$$

In the second stage, the lactate was oxidized to acetate and CO_2. Strominger (173) has observed, in extracts of *Staphylococcus aureus*, *E. coli*, and *A. aerogenes*, a direct enzymatic transfer of enolpyruvate from phosphoenol pyruvic acid to position 3 of acetyl-glucosamine linked to the nucleotide UDP-glucosamine:

$$\text{UDP-acetyl glucosamine} + \text{phosphoenol pyruvic acid} \rightarrow \text{UDP-acetyl-glucosamine-pyruvate} + \text{inorganic phosphate} \qquad 25.$$

UDP-acetyl-glucosamine-pyruvate is probably the precursor of uridine diphosphoglucose-lactic ether, one of the nucleotides which accumulates in penicillin-inhibited *Staphylococcus aureus*. The enzyme which catalyzes the transfer of pyruvate has been called pyruvate UDP-acetyl-glucosamine transferase. James & Slater (174) have studied the metabolism of ^{14}C-labelled pyruvate in apple tissue and in root tips of barley. After incubation, the radioactivity was recovered in CO_2, in a series of carboxylic acids, and in the amino acid alanine, aspartic acid, glutamic acid, γ-amino butyric acid, glutamine, and asparagine; no radioactivity was present in the sugars or polysaccharides. Beloff-Chain and co-workers (175) have followed the fate of pyruvate-2-^{14}C in brain slices and liver slices from normal and alloxan dia-

betic rats by use of the techniques previously mentioned (69, 70). In brain slices, pyruvate was metabolized to CO_2, lactic acid, alanine, aspartic acid, glutamic acid, and γ-amino butyric acid. In liver slices, pyruvate was transformed into glycogen, oligosaccharides, maltose, lactic acid, CO_2, glucose β-hydroxybutyric acid, alanine, glutamic acid, and some carboxylic acids. The amount of glycogen synthesized from pyruvate was about the same as that synthesized under similar experimental conditions from glucose and was less than that synthesized from fructose both in normal and diabetic livers. These results suggest that pyruvate is incorporated into glycogen in a reversal of the glycolytic pathway.

Dickens & Williamson (176) have studied the metabolism of ^{14}C-labelled hydroxypyruvate in the intact rat and in rat liver slices, and have shown that this substance, both *in vivo* and *in vitro*, was rapidly incorporated into liver glycogen. The distribution of radioactivity in the glucose molecule suggested that the incorporation of hydroxypyruvate into glucose occurred symmetrically with the combination of two unbroken carbon chains by a fairly direct route, which did not pass through L-serine and probably not through pyruvate as intermediates. The authors have suggested, on the basis of their results, possible pathways of serine and hydroxypyruvate metabolism; however, conclusive evidence is not yet available.

By using deuterium-labelled lactate, Hoberman (177) has shown that glucose obtained by the hydrolysis of liver glycogen following the injection of DL-α-deuterio lactate into rats contained little or no deuterium in positions 1, 2, 3, or 5; the highest concentration was in position 4, and a relatively high concentration in position 6. The presence of deuterium in position 4 is in accordance with the view that the α-hydrogen atom of lactate is a precursor of the hydrogen in position 1 of phosphoglyceraldehyde. As fructose diphosphate is formed from two molecules of triosephosphate, it would be expected that the glucose molecule would be labelled in position 3. As this was not so, it was suggested that there had been an exchange reaction with the hydrogen of the body water. The relatively high concentration of deuterium found in position 6 was caused by the utilization of the α-hydrogen atom of lactate in another reduction step probably involving the reductive synthesis of malate.

Noble, Reed & Wang (178), have followed the utilization of ^{14}C specifically labelled pyruvate, acetate, and CO_2 in *Penicillium digitatum* in a series of time-course experiments and showed that pyruvate was utilized by way of acetate and that the latter was metabolized via the tricarboxylic acid cycle. Olson & Chain (179) have studied the organic acid metabolism of acetate grown *Penicillium chrysogenum* and showed that washed mycelial suspensions oxidized acetate but no other organic acid of the tricarboxylic acid cycle. The authors stated that, because of the problems of permeability, no deduction could be made in this study on the major pathway of acetate oxidation in the intact cells of *P. chrysogenum*. In a second paper, however (180), the authors incubated methyl-labelled ^{14}C-acetate with mycelial suspensions or cell-free extracts of *P. chrysogenum*, and the fate of the ^{14}C was followed. In

fresh mycelial preparations, 40 per cent of the utilized acetate appeared in glutamic acid. The labelling pattern of the glutamate indicated its formation by a citrate condensation mechanism. The presence of several citric acid cycle enzymes was demonstrated, but no accumulation of citrate from acetate and oxalacetate occurred in fortified cell-free extracts. Direct evidence was given in support of the hypothesis that the ineffectiveness of malonic acid as an inhibitor of acetate oxidation in *P. chrysogenum* can be attributed to its inability to enter the cell.

Glasky & Rafelson (181) have carried out kinetic studies on a strain of *E. coli* adapted to growth on acetate as the sole carbon source. The organisms were incubated for varying time periods with acetate-1-^{14}C and acetate-2-^{14}C, and the distribution of incorporated radioactivity into the various metabolites was followed. From their results, they concluded that the pathway of carbon in this system is as follows:

$$\text{Acetate} \rightarrow \text{acetyl coenzyme A} \rightarrow \text{succinate} \qquad 26.$$

Isocitratase, which catalyzes the aldocleavage of isocitrate to succinate and glyoxylate, is thought to play an essential role in the biosynthesis of cell constituents of microorganisms growing on acetate as sole carbon source. Kornberg, Gotto & Lund (182) have measured the activity of this enzyme in *Pseudomonas ovalis* grown on different growth substrates and have shown that isocitratase was found in appreciable amounts only in the presence of acetate. Madsen & Hochster (183) have found similar results with cell-free extracts of *Xanthomonas phaseoli*. In a series of papers on the biosynthesis of cell constituents from C_2 compounds, it has been found that, when pseudomonas was grown on glycollate (184), glycine (185), or oxalate (186), glyoxylic acid was found. As under these conditions, the activity of isocitratase was negligible; another route (or routes) must exist for the synthesis of the tricarboxylic acid cycle intermediates from glyoxylic acid. Olson (187) has identified isocitratase in crude extracts of the mold *P. chrysogenum Q 176*, and has partially purified this enzyme from bakers' yeast. The biological function of the enzyme was discussed, and a possible mechanism for the cleavage of isocitrate involving chelation with magnesium was proposed.

Lundquist *et al.* (188) have carried out a study on the anaerobic metabolism of acetaldehyde in rat liver suspensions and found that the acetaldehyde was transformed rapidly into ethanol and acetate. The addition of pyruvate increased by about 50 per cent the formation of acetate.

Alternative Pathways of Carbohydrate Metabolism

Recent work on carbohydrate metabolism in plants has been reviewed in detail by Gibbs (189).

Pentose phosphate pathway.—Excellent reviews covering the present state of knowledge of the pentose phosphate pathway have been written by Dickens (190) and by Glock & McLean (191); the latter is limited to a discussion of the results obtained with mammary gland tissue.

The existence of the pentose phosphate pathway of carbohydrate metabolism has been demonstrated in extracts of tobacco leaves and seedlings (192) in the midgut of the silkworm *Bombyx mori* (193), *Acetobacter suboxydans* (194), *Aspergillus niger*, (195), *Nocardia opaca* (196), smooth stomach muscle of the pigeon (197), homogenates of rat brown adipose tissue (198), bovine cornea and lens (199), and in neoplastic cell tissue (200). Quantitative estimations of the participation of the pentose phosphate cycle in glucose metabolism, deduced from the distribution of ^{14}C derived from specifically labelled glucoses, are, however, difficult, because of the randomization of carbons and the dilution of the intermediates by their concomitant formation via other metabolic pathways. Wood & Katz (201) calculated the theoretical extent to which the recycling of hexose phosphate via the pentose cycle causes the randomization of ^{14}C derived from specifically labelled ^{14}C glucose.

Couri & Racker (202) have shown that, in a reconstituted enzyme system containing the purified enzymes of the pentose phosphate cycle, glucose-6-phosphate was completely oxidized to CO_2. In this system, oxidized glutathione, in the presence of glutathione reductase, or oxygen, in the presence of the old yellow enzyme, acted as hydrogen acceptors. The addition of the glycolytic enzymes produced an inhibition of glucose oxidation by the pentose phosphate cycle in the above system; the inhibition was caused by a competition between the two pathways for the intermediates common to both cycles, i.e., glucose-6-phosphate, fructose-6-phosphate, fructose diphosphate, and triose phosphate. The authors suggested a new route to fructose-6-phosphate in their plurienzyme system, involving the participation of aldolase, transaldolase, and sedoheptulose diphosphatase, according to the scheme:

Glyceraldehyde-3-phosphate + sedoheptulose-7-phosphate
→ erythrose-4-phosphate + fructose-6-phosphate 27.

Dihydroxyacetonephosphate + erythrose-4-phosphate
→ sedoheptulose diphosphate 28.

Sedoheptulose diphosphate → sedoheptulose-7-phosphate + inorganic phosphate 29.

Sum: 2 triose phosphate → fructose-6-phosphate + inorganic phosphate 30.

Jolley, Cheldelin & Newburgh have found that the pentose cycle was about four times as active in foetal pig heart homogenates (203) and in a soluble enzyme fraction from pig foetal heart (204) than in similar preparations from the adult pig heart. McLean (205) has shown that, in rat mammary gland during the period of lactation, the $^{14}CO_2$ formation from glucose-1-^{14}C increased, whereas the $^{14}CO_2$ formation from glucose-6-^{14}C remained constant; the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase increased during the lactation period and returned to normal during the period of mammary involution. These results showed clearly that, during the physiological state of lactation, the glucose is preferentially oxidized via the pentose cycle.

Sedoheptulose-7-phosphate has been isolated from rat (206) and calf liver (207). Sie, Nigam & Fishman (208) have shown that there exists an enzymic system in the supernatant solution of a rat liver homogenate which can form sedoheptulose-7-phosphate from adenosine by a mechanism not yet elucidated. Bonsignore, Pontremoli & Grazi (209) have carried out further experiments to elucidate the mechanism whereby glucose-6-phosphate, when added as substrate to rat liver homogenates, was metabolized to triose phosphates without the addition of ATP and TPN. As the participation of the glycolytic cycle or pentose shunt pathway was thus excluded, the authors suggested that there is either a mono-oxidative heptoformation in this system, the mechanism of which has still to be clarified, or aldolase acceptors are present in catalytic amounts. Dische (210) has also reported the transformation of glucose-6-phosphate into sedoheptulose-7-phosphate, ribose phosphate, ketopentose phosphate, triose phosphates, and erythrose-4-phosphate in an exhaustively dialyzed hemolysate in which the presence of triose phosphates and fructose-6-phosphate, even in catalytic amounts, was excluded. The non-oxidative formation of sedoheptulose-7-phosphate has also been demonstrated in skeletal muscle by Moret & Sperti (211, 212, 213).

Schramm, Klybas & Racker (214) have partially purified, from *Acetobacter xylinum*, a fructose-6-phosphate phosphoketolase which catalyzes the phosphorolytic cleavage of fructose-6-phosphate into erythrose-4-phosphate and acetylphosphate.

Biosynthesis of shikimic acid from glucose.—Glucose can be transformed into cyclic compounds through reactions involved in the pentose phosphate shunt and the glycolytic cycle. It is now generally accepted, from enzymic studies (215, 216), that 5-dehydroquinic acid and 5-dehydroshikimic acid are essential intermediates in the biosynthesis of the aromatic amino acids, tyrosine, phenylalanine, and tryptophan, from glucose. Srinivasan, Katagiri & Sprinson (217) have now definitely established that, in mutant strains of *E. coli*, the pathway from sedoheptulose-1-7-diphosphate [formed from sedoheptulose-7-phosphate and hexose diphosphate (218)] to 5-dehydroquinic acid (219) involved cleavage of sedoheptulose diphosphate to erythrose-4-phosphate and dihydroxyacetone phosphate (218), conversion of dihydroxyacetone phosphate to phosphoenol pyruvic acid and synthesis of 5-dehydroquinate from erythrose-4-phosphate and phosphoenol pyruvic acid. The intermediate product of this last reaction has been shown to be 2-keto-3-deoxy-D-arabo-heptonic-acid-7-phosphate; Srinivasan & Sprinson (220) have succeeded in purifying, from extracts of *E. coli* mutants, the enzyme which catalyzes the condensation of erythrose-6-phosphate and phosphoenol pyruvic acid. The name "2-keto-3-deoxy-arabo-heptonic-acid-7-phosphate synthetase" was suggested for this enzyme. The reaction appeared to be irreversible, not to require any cofactors, and to be specific for erythrose-6-phosphate and phosphoenol pyruvic acid.

Weissbach & Hurwitz (221) have demonstrated the formation and accumulation of 2-keto-3-deoxyheptonic acid in crude cell-free extracts of *E.*

coli strain B incubated with ribose-5-phosphate and have also prepared, from *E. coli*, an enzymic fraction which catalyzed the formation of 2-keto-3-deoxyheptonic acid both from ribose-5-phosphate and from erythrose-4-phosphate and phosphoenol pyruvic acid (222). These enzyme fractions also contained a phosphatase activity which yielded inorganic phosphate from 2-keto-3-deoxy-arabo-heptonic-acid-7-phosphate. The occurrence of 2-keto-3-deoxyheptonic acid in the *E. coli* strain strongly supports the suggestion of Srinivasan *et al.* for the mechanism of formation of shikimic acid. Weissbach & Hurwitz found that, in *E. coli*, 2-keto-3-deoxyheptonic acid, which does not appear to be a precursor of dehydroquinic acid, was formed in large amounts and was rapidly transformed into another intermediate not as yet identified. Levin & Racker (223), incubating ribose-5-phosphate and phosphoenol pyruvic acid in the presence of crude extracts of *Pseudomonas aeruginosa*, have demonstrated the formation of 2-keto-3-deoxy-8-phosphooctonic acid.

Phosphoenol pyruvic acid + ribose-5-phosphate
→ 2-keto-3-deoxy-8-phosphooctonic acid **31.**

They have partially purified the enzyme 2-keto-3-deoxy-8-phosphooctonic synthetase from this preparation and have shown that 2-keto-3-deoxy-8-phosphooctonic acid, unlike 2-keto-3-deoxyphosphoheptonic acid is not a precursor of dehydroquinic acid.

The formation of 3-ketoglycosides as intermediate in the bacterial catabolism of disaccharides has been reported by Bernaerts & de Ley (224). The catabolism of lactose and lactobionate by a strain of *Corynebacterium simplex* was shown to give rise to 4-O-β-3-keto-D-galactoside-D-glucose and 4-O-β-3-keto-D-galactoside-D-gluconate respectively. These findings demonstrate a new pathway of disaccharide catabolism in bacteria involving oxidation in the glycosyl residue and provide the first example of 3-keto sugars isolated as intermediates in a metabolic pathway.

Glucuronic acid pathway.—Studies, *in vivo*, on the biosynthesis of ascorbic acid and of L-xylulose led to the postulation of an alternative cyclic pathway of glucose metabolism via D-glucuronic acid, L-gulonic acid, and L-xylulose; the latter enters the pentose phosphate cycle and is converted to glucose (118). Further evidence for the glucuronic acid cycle has been obtained by Eisenberg, Dayton & Burns (225) from experiments in which D-glucuronolactone-1-^{13}C, 6-^{14}C or L-gulonolactone-6-^{13}C, 1-^{14}C were injected into rats and guinea pigs. The liver glycogen was found to be radioactive, and the isotope distribution in the glucose molecule derived from it conformed with what would be expected were the glucuronic acid cycle active. A number of studies on isolated systems have been carried out to elucidate further the mechanism of formation of the intermediates of the glucuronic acid cycle and to identify the intermediate products. It is now generally agreed that, in microorganisms, the first product of metabolism of glucuronic acid is a keturonic acid. It has, been confirmed, in fact, by a number of investigators working with *E. coli*, (226, 227), *Serratia marcescens* (228),

Erwinia carotovora (229), *A. aerogenes* (230), and other bacteria (231), that glucuronic acid is transformed into 5-keto-L-gulonic acid (or D-fructuronic acid) and galacturonic acid into 5-keto-L-galactonic acid (or D-tagaturonic acid) by the enzyme uronic acid isomerase.

There is, however, some difference of opinion as to the nature of the subsequent products of metabolism, the hexonic acids, which are formed by the reduction of the keturonic acid, the reactions being catalyzed by keturonic reductase in the presence of DPNH or TPNH. Whereas McRorie, Williams & Payne (231) have reported that, by the reduction of fructuronic and tagaturonic acids, L-gulonic and L-galactonic acid were obtained respectively, Kilgore & Starr (232) have demonstrated, contrary to their earlier findings (229), that the acids formed in this reaction were not L-gulonic and L-galactonic acid, but D-mannonic and D-altronic acids. These latter acids have been identified with specific and accurate analytical methods by Ashwell, Wahba & Hickman (233) working with *E. coli;* these authors have, in addition, identified 2-keto-3-deoxygluconic acid as a subsequent enzymic reaction product common to both D-mannonic and D-altronic acid in *E. coli.* The authors showed that 2-keto-3-deoxygluconic acid was converted to pyruvic acid and triose phosphate, giving a stoichiometric yield in the presence of ATP, and a dialysed enzyme preparation from the *E. coli* cells grown on glucuronic acid. The reaction is similar to that described previously by de Ley & Doudoroff (234) in their work on the metabolism of *P. saccharophila.* McRorie & Novelli (230), in preliminary experiments with glucuronate-adapted *A. aerogenes,* have demonstrated that fructuronate is first phosphorylated by ATP and that the fructuronate-1-phosphate is then split into dihydroxyacetone phosphate and tartronic semialdehyde, which are then transformed into pyruvate and the latter converted to acetate, hydrogen, and carbon dioxide. Kilgore & Starr (235) have demonstrated a different pathway of glucuronic acid and galacturonic acid metabolism that appears to occur in the phytopathogenic pseudomonas species, in which the initial product of the enzymic oxidation of D-galacturonic acid has been identified as mucic acid. Presumably, glucaric acid would be formed from glucuronic acid.

In studies on the kinetics of growth of some bacteria of the species enterobacteriaceae, Hollmann & Thofern (236) have concluded that glucuronic acid is metabolized via the corresponding lactone. In homogenates of rat kidney, on the other hand, it has been demonstrated by Dowben (237) that, by using ^{14}C-labelled glucurone lactone or glucuronic acid, more $^{14}CO_2$ was formed from the former than the latter. Mano *et al.* (238) have reported the presence, in rat liver, of a TPN-dependent gulonic dehydrogenase which transforms D-glucuronolactone to L-gulonolactone. The enzyme L-gulonic acid (DPN) dehydrogenase, which catalyzes the transformation of L-gulonic acid into L-xylulose, has been found in rat kidney (239) and has been purified from hog kidney extracts (240). Further evidence was obtained for the role of 3-keto-L-gulonic acid as an intermediary product. This product, however,

does not appear to be an intermediate in the biosynthesis of ascorbic acid, as previously believed; in fact, it has now been shown by three different groups of authors that, in a soluble enzyme from rat liver microsomes, the presence of L-gulono-γ-lactone is required in order to obtain ascorbic acid biosynthesis (241, 242, 243). It has been shown that the lactone was formed from gulonic acid by the enzyme lactonase (242), and 2-keto-L-gulonolactone is believed to be an intermediate in ascorbic acid biosynthesis (243). Chatterjee *et al.* (244) have, in fact, identified this substance as an intermediate in the transformation of L-gulonolactone into ascorbic acid by a soluble enzyme preparation obtained from goat liver microsomes. Bublitz & Lehninger (241) have suggested that the enzyme responsible for the oxidation of L-gulono-γ-lactone is probably similar to the auto-oxidizable flavoprotein found in plants (245), which, in the presence of oxygen, oxidizes L-gulono-γ-lactone to ascorbic acid. The biosynthesis of ascorbic acid via gulono-γ-lactone has also been shown to occur in strawberries by Loewus, Finkle & Jang (246); in fact, it has been shown that, when the glucuronolactone was labelled with ^{14}C in C-1, the radioactivity was recovered in C-6 of ascorbic acid. In addition to the pathway of ascorbic acid formation from glucuronic acid via L-gulono lactone, there appears to be (246) another possible pathway of ascorbic acid formation; thus, when the plants were stem fed glucose-6-^{14}C, the radioactivity was recovered in C-6 of the ascorbic acid molecule. The mechanism of this pathway of ascorbic acid synthesis, which involved the epimerization of carbon atom 5, remains to be elucidated. When ascorbic acid-6-^{14}C was stem fed into strawberry plants, the ^{14}C was recovered in the carbon atoms 1 and 6 of the glucose moiety of sucrose; the mechanism of this transformation is unknown. Dayton, Eisenberg & Burns (247) have found similar results in studies on the distribution of ^{14}C in the glucose molecule obtained by hydrolysis of liver glycogen in guinea pigs that had been injected with ascorbic acid, dehydroascorbic acid, or diketogulonic acid labelled in carbon atoms 1 or 6. The recovery of the radioactivity on the terminal carbon atoms of the glucose molecule suggested that the ascorbic acid was broken down into triose units.

The experiments of Burns *et al.* (248) suggested that inositol also participates in the glucuronic acid cycle; these authors found that uniformly labelled ^{14}C or randomly labelled tritium inositol were transformed into labelled D-glucuronic acid and L-gulonic acid in rats both *in vivo* and in kidney extracts. *In vitro*, no radioactivity was found in L-glucuronic acid, contrary to the results previously obtained by Charalampous and his colleagues (249, 250). Richardson & Axelrod (251), working with whole homogenates of rat kidney, found that the $^{14}CO_2$ formed from myoinositol-6-^{14}C was not decreased by the addition of non-radioactive D-glucuronate, D-glucuronolactone, or L-gulonolactone; these findings suggest another pathway for the oxidation of inositol. In this connection, Moscatelli & Larner (252) have observed that injecting ^{14}C-labelled myoinositol into rats caused a rapid formation of $^{14}CO_2$ corresponding to about 25 per cent of the product in-

jected, whereas only a small fraction of the product was incorporated into liver glycogen.

Entner-Doudoroff pathway.—It has been shown by Wang, Stern & Gilmour (253) that, in *Pseudomonas aeruginosa*, *Pseudomonas reptilivora*, and *Pseudomonas fluorescens KB1*, gluconic acid was metabolized entirely via the Entner-Doudoroff pathway, whereas glucose was metabolized also via the pentose cycle. An enzymic system capable of oxidizing 2-deoxy-D-glucose to 2-deoxy-D-gluconic acid has been isolated from sonic extracts of *P. aeruginosa* (254). Doi, Halvorson & Church (255) have shown, by kinetic studies on the formation of pyruvate from 2-keto-6-phosphogluconic acid and from 6-phosphogluconic acid in cell-free extracts of spores of *Bacillus cereus*, that 2-keto-6-phosphogluconic acid was converted to pyruvate directly without passing through 6-phosphogluconic acid. De Ley & Stouthamer (256) have found that in *A. suboxidans* gluconate is oxidized by three enzymes: two TPN dependent soluble dehydrogenases, one of which forms 2-ketogluconate and the other, 5-ketogluconate, and a particle-linked coenzyme independent oxidase which forms 2-ketogluconate. The further metabolism of 2-ketogluconate was the same as that which has been previously described in *Corynebacterium helvolum* (257); that is to say, in the absence of 2-ketoglucokinase, it was reduced to gluconic acid, which was phosphorylated by glucokinase and the 6-phosphogluconic acid thus formed was further metabolized by the hexose monophosphate shunt. A specific adaptive enzyme, 2-keto-D-gluconokinase, has been isolated by Ciferri, Blakley & Simpson (258) from the cells of *L. mesenteroides* grown on 2-keto-D-gluconate; the product of this enzymic reaction has been isolated and identified as 2-keto-6-phospho-D-gluconate.

A new pathway of glucose metabolism.—The possibility of a hitherto unknown pathway of glucose metabolism has been suggested by the experiments of Serif & Wick (259) on the competitive inhibition of glucose oxidation by 6-deoxy-6-fluoroglucose in rat kidney slices. These authors have found that 6-deoxy-6-fluoroglucose inhibited the oxidation of uniformly labelled ^{14}C glucose and fructose to CO_2, whereas it had no effect on the oxidation of lactate-1-^{14}C or acetate-1-^{14}C; this is in agreement with the findings of Blakley & Boyer (260) with yeast preparations. Serif & Wick have made the interesting observation that increasing the concentration of 6-deoxy-6-fluoroglucose did not increase the inhibition of CO_2 formation. This is interpreted by the authors as indicating that 6-deoxy-6-fluoroglucose inhibited a specific pathway of glucose metabolism while leaving intact the other pathways. Studies with glucose labelled with ^{14}C in carbon atoms 1 or 6 indicated that 6-deoxy-6-fluoroglucose acted as an inhibitor at some stage before the formation of glucose-6-phosphate. Thus glucose must be oxidized with the loss of CO_2 to an intermediate so far unidentified or, alternatively, it could be phosphorylated on carbon atom 1. The same results as those obtained in kidney slices have also been obtained by Serif *et al.* (261) in rat epididymal adipose tissue and rat diaphragm muscle.

Carbon Dioxide Fixation

In a review article on carbon dioxide fixation, Utter (262) discussed two reactions, one catalyzed by the "malic enzyme" and the other by phosphopyruvate carboxykinase (oxalacetate decarboxylase). Corwin (263) has isolated and partially purified from mitochondria of rat liver an enzyme which catalyzes the formation of CO_2 and pyruvate from oxalacetate. This enzyme was not dependent on a nucleotide cofactor and differs in this way from other oxalacetic decarboxylases isolated from chicken and lamb liver which are stimulated by inosine triphosphate (264, 265). Saz & Vidrine (266) have shown that, in muscle strips from the parasitic intestinal helminth *Ascaris lumbricoides* (which has a predominantly anaerobic metabolism), in which succinate accumulates as an end product of carbohydrate metabolism, the succinate was formed by carboxylation of pyruvate followed by reduction of the product to succinate, a reaction similar to that described by Wood & Werkman in propionibacterium (267, 268). It was not evident from the results whether the fixation of CO_2 into pyruvate was catalyzed by the "malic enzyme" or "oxalacetic decarboxylase"; however, an active "malic enzyme" has been partially purified from *A. lumbricoides* muscle. The succinate formed accumulates but is also partly decarboxylated directly to propionate by an enzyme system similar to that previously described in bacteria.

Stoppani, Conches & de Favelukes (269) have reported that CO_2 fixation in two types of yeast (*Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae*) occurred by the carboxylation of phosphoenol pyruvic acid to give oxalacetate. $^{14}CO_2$ assimilation was shown to take place in the presence of acetate, acetaldehyde, pyruvate, and glucose, and ^{14}C was recovered mostly in aspartic acid, glutamic acid, malic acid, and tricarboxylic acids. The importance of carbon dioxide as a metabolite was discussed by the authors; they point out that the carboxylation reaction provides the main supply of oxalacetate carbon utilized in amino acid synthesis and replaces the oxalacetate destroyed by a decarboxylase. Under anaerobic conditions in the presence of glucose substrate, a greater amount of ^{14}C was recovered in malic acid, fumaric acid, succinic acid, and aspartic acid. Extracts of *Clostridium butyricum* have been shown to catalyze the rapid exchange between CO_2 and the carboxyl group of pyruvate (270, 271). The mechanism of CO_2 activation in this system has been studied by Mortlock, Valentine & Wolfe (272). Two protein fractions prepared from the bacterial extracts were required for this reaction and the phosphate requirement in this system could be replaced by coenzyme A in catalytic amounts. The exact function of coenzyme A was not clear, but the poor rate of exchange of acetyl coenzyme A and pyruvate showed that it did not act as an intermediate in the exchange reaction. Experiments on acetoin formation, by adding free acetaldehyde to the system, suggested that the active 2-carbon compound was at the aldehyde level of oxidation. In experiments (273) in which either acetyl-1-^{14}C-phosphate or $NaH^{14}CO_3$ were present in the test system, the incorporation of acetyl phosphate into pyruvate was detected in extracts of *C. butyricum* if sufficient

reducing potential was provided, as, for example, by the addition of hydrosulphite to the system. The incorporation of ^{14}C-labelled carbon dioxide in the dark into several organic and amino acids has been shown to occur by the carboxylation of phosphoenol pyruvic acid in excised leaves of *Nicotiana tabacum* (274) and of *Bryophyllum calycinum* (275). No ^{14}C was found in carbohydrates or in the phosphorylated sugars.

Stanley, Young & Graham (276) have shown that germinating pine pollen incorporated carbon dioxide into the organic acids, amino acids, and protein fractions. The incorporation of $^{14}CO_2$ into sugars by the cotyledons from etiolated sunflower seedlings kept in the dark has been reported by Bradbeer (277). He found that, in experimental periods exceeding 5 min., sucrose was the most heavily labelled compound; within shorter periods, the bulk of the radioactivity was recovered in malate and related acids and amino acids, suggesting that $^{14}CO_2$ was incorporated by a β-carboxylation of pyruvate or phosphoenol pyruvic acid to give malate either directly or indirectly by way of oxalacetate. Bradbeer also discussed a probable pathway of conversion of malate to sucrose via a reversal of the glycolytic reactions and the possible role of malate as an intermediate in the conversion of fat to carbohydrate in the cotyledons.

Jolchine (278) has studied the distribution of ^{14}C in the malic acid molecule after $^{14}CO_2$ fixation in leaves of *Bryophyllum daigremontianum*. Several hypothesis on the mechanism of biosynthesis of malic acid are discussed by the author on the basis of her results. Suzuki & Werkman (279) have extended their work on CO_2 fixation in *Thiobacillus thiooxidans* and have shown that cell-free preparations of this chemoautotrophic organism contain the necessary enzymes for fixation of CO_2 and synthesis of carbohydrates from CO_2 through a cyclic mechanism similar to that of photosynthesis. McFadden (280) has studied some products of CO_2 fixation by *Hydrogenomonas facilis*. He showed that autotrophic assimilation of $^{14}CO_2$ for 25 or 45 sec. produced labelled phosphoglyceric acid, fructose-6-phosphate, sedoheptulose-7-phosphate, and ribulose diphosphate. Under heterotrophic conditions, the same compounds were labelled to a lesser extent. On the basis of his findings, McFadden concluded that *H. facilis* probably assimilates CO_2 autotrophically by a pathway similar to that operating in photosynthesis. Alanine, aspartic acid, glutamic acid, glycine, serine, and threonine were found to be products of 1 min. fixation under autotrophic conditions, whereas glutamic acid was a major product of 1 min. heterotrophic fixation.

In studies by Quayle & Keech (281) in which the incorporation of carbon from ^{14}C formate or ^{14}C bicarbonate was followed in cultures of *Pseudomonas oxalaticus* grown on formate, they showed that at least 94 per cent of the carbon passed through the stage of carbon dioxide (or a compound in ready equilibrium with it), and the organism synthesized cell constituents from carbon dioxide by a cycle similar to that found in photosynthetic and some other autotrophic organisms. The necessary energy for the organism was presumably derived from the oxidation of formate to carbon dioxide. Con-

firmation of the participation of a carbon dioxide fixation cycle similar to that found in photosynthesis was provided by experiments carried out with cell-free extracts of *P. oxalaticus* grown on formate (282). The authors, using ^{14}C formate or ^{14}C bicarbonate, demonstrated the presence of 2 enzymes in the extracts peculiar to the photosynthetic cycle: phosphoribulokinase and carboxydismutase. Moses, Holm-Hansen & Calvin (283) have followed the process of non-photosynthetic $^{14}CO_2$ fixation in two algae, *Chlorella pyrenoidosa* and *Nostoc muscorum*, and in the mold *Zygorrhynchus moelleri* by using their method of radiopaperchromatographic technique after varying time intervals. With the two algae, they found the only carboxylation reaction of importance in the dark was the carboxylation of pyruvate to oxalacetate. The ^{14}C was also recovered in some of the phosphorylated derivatives, probably because of a reversal of glycolysis. In the mold *Z. moelleri*, carbon dioxide was also incorporated by the carboxylation of pyruvate, but the ^{14}C did not appear to enter the hexose monophosphate by a reversal of glycolysis but rather by a reversal of the decarboxylation of 6-phosphogluconic acid.

Hammen & Wilbur (284) have demonstrated that oysters incorporated $NaH^{14}CO_3$ from sea water into calcium carbonate and the protein matrix of shell. By incubating mantle tissue with labelled compounds, it was shown that carbon dioxide combined directly with propionate to form succinate, a mechanism which has been previously described by Lardy & Adler (285) to occur in liver mitochondria.

Photosynthesis

Gibbs & Cynkin (286) have studied the conversion of ^{14}C-labelled carbon dioxide to starch-glucose during photosynthesis by spinach chloroplasts and found the same type of asymmetric labelling pattern as that found by Gibbs & Kandler (287) in glucose formed from carbon dioxide in intact cells. The authors concluded that this similarity further strengthens the concept that the isolated structure possesses a typical photosynthetic carbon cycle. Kandler & Gibbs (288) have incubated glucose-1-^{14}C, 2-^{14}C, and 6-^{14}C with chlorella suspensions in the light and in the dark and have determined the distribution of ^{14}C carbon in the glucose units of the polysaccharide formed. They concluded that the quantitative results obtained cannot be explained by a recycling of glucose involving the aldolase and transketolase reactions.

Glycolate is known to be an important intermediate in photosynthesis; it becomes rapidly labelled when photosynthesis occurs in the presence of $^{14}CO_2$ and is an intermediate in the formation of glycine and serine. It has been suggested by Wilson & Calvin (289) and Weissbach & Horecker (290) that glycolate is formed from the ribose phosphate of the photosynthetic cycle. Griffith & Byerrum (291), using detached leaves from three-months-old tobacco plants (*Nicotiana rustica*), have shown that, by vacuum infiltration of the leaves with an aqueous solution of D-ribose-1-^{14}C, the ^{14}C was recovered in glycolic acid, glycine, serine, and alanine. The ^{14}C was found

principally in the alpha carbons of glycolate and of glycine, in the alpha and beta carbons of serine, and in the beta carbon of alanine. There was a much greater incorporation of ^{14}C when the leaves were exposed to the light, but the relative distribution in the above compounds was similar in the light and in the dark. Moses & Calvin (292) have studied the incorporation of tritium from tritiated water in chlorella cells grown under conditions of light and dark. They found that the substances incorporating tritium were essentially the same as those which had been previously shown to incorporate ^{14}C from $^{14}CO_2$. About three times as much tracer was fixed in the light than in the dark; the compounds formed were similar, although, in the dark, a greater percentage of the tritium incorporated appeared as amino acids. During the shortest incubation period in light, the tritium was incorporated mainly in sugar monophosphates, phosphoglyceric acid, glutamic acid, and malic acid. The labelling of glycolic acid appeared very early, and it was, in fact, the most active compound produced. The authors suggested a scheme for hydrogen transport involving the alternate oxidation and reduction of glycolic acid. The difficulties of interpreting the results of biochemical studies with hydrogen isotopes caused by non-specific exchange reactions were discussed. 2-Carboxy-4-ketopenitol diphosphate had previously been tentatively identified as one of the compounds obtained from $^{14}CO_2$ by suspensions of photosynthesizing chlorella cells, but the significance of this finding remained uncertain, as this compound did not penetrate the cells.

Moses and Calvin (293) have now briefly reported that when a chloroplast sap preparation from spinach leaves was incubated for 30 minutes in the light with ^{14}C-labelled 2-carboxy-4-ketopenitol diphosphate; 19 per cent of the ^{14}C was recovered in the original compound, 9 per cent as the corresponding monophosphate ester, and 72 per cent as a new compound. Properties of this new substance showed that it could be a saccharic acid or a saccharinic acid. The physiological significance of this conversion was not clear. The chloroplast sap preparation used in this work formed intermediates of the reductive cycle in the presence of ribulose diphosphate or phosphoglyceric acid, whereas 2-carboxy-4-ketopentitol diphosphate was not converted into any of the cycle intermediates. The authors concluded, therefore, that it probably does not participate in the photosynthetic carbon cycle. The purification and properties of a phosphatase from *Spinacia oleracea*, which specifically hydrolyzed the carbon-1-phosphate group of both ribulose diphosphate and fructose diphosphate, has been described by Chakravorty, Chakrabortty & Burma (294). The authors discussed the possible importance of this enzyme in the photosynthetic cycle and speculated that it may be the controlling enzyme for the ribulose diphosphate pool by forming ribulose-5-phosphate. The enzyme could also have a second function in the photosynthetic cycle in the conversion of fructose diphosphate into fructose-6-phosphate. In a brief note, Moses & Calvin (295) have given preliminary evidence, using their radiopaperchromatographic techniques, for the occurrence of labelled erythrose-4-phosphate in algae (*Chlo-*

rella pyrenoidosa) that was allowed to carry on photosynthesis in the presence of $^{14}CO_2$. The possible role of erythrose-4-phosphate in the photosynthetic cycle had been previously proposed by Bassham *et al.* (296) but has not been hitherto detected to occur in any plant. Holm-Hansen and co-authors (297) have studied the effect of various mineral salts, both singly and in combination, on the uptake and distribution of photosynthetically incorporated carbon 14 in chlorella. The authors pointed out that most of the previous work on photosynthesis in their laboratory was undertaken with algae suspended in various dilute buffer solutions or in distilled water alone on the assumption that the salts remaining within the cells from the time of growth in nutrient solution were sufficient in quantity that the cells should not become deficient in one of the essential elements during the course of the experiment. However, since some evidence has been reported showing that the addition of salts to algae suspended in distilled water could effect the metabolic system of the plant, the present investigation was undertaken. The authors suggested from the results obtained that the salts, while influencing some metabolic pathways, did not greatly affect the photosynthetic cycle.

Hormones

Insulin.—Many investigators are now of the opinion that both the permeability theory and the hexokinase theory of insulin action, which have received so much attention in recent years, are inadequate to explain all the diverse known effects of insulin, and new theories as to the mode of action of the hormone have been advanced.

Chain, Beloff-Chain & Pocchiari (298), in a review on the mechanism of the mode of action of insulin, discussed in detail results of other authors and of their own experiments that cannot be reconciled with the permeability theory or hexokinase theory of insulin action. They advanced the hypothesis that insulin must have a more general action resulting in the stimulation of a number of synthetic reactions, not always involving glucose. They suggested that insulin may act by raising the energy potential of the cell, enabling it to perform a larger amount of energy requiring metabolic reactions. More recently, Beloff-Chain and co-authors (116) have extended this theory and have proposed that the TPN/TPNH system is specifically affected by insulin. The authors referred to the function of the TPN/TPNH in fat synthesis and in the oxidative pathway of glucose metabolism (the pentose shunt) and discussed the known insulin effects on these reactions. Further support for the hypothesis that the TPN/TPNH system is influenced by insulin is given by the work of McLean (299), who has followed up the experiments of Abraham, Cady & Chaikoff (300) with mammary gland tissue and has shown that the stimulation by insulin of a reductive synthetic process utilizing TPNH, such as fatty acid synthesis, could lead to a secondary increase in the pentose pathway of glucose oxidation. She studied the oxidation of glucose-1-^{14}C and glucose-6-^{14}C in rat mammary gland slices at different functional stages and found, in agreement with Abraham *et al.* (300), that, in the lactating gland,

the oxidation of C-1 of glucose to CO_2 was greatly stimulated by insulin, whereas no insulin effect was found with the glands from pregnant animals. The author suggested that this difference was related to the difference in fatty acid synthesis in the gland during lactation and during pregnancy. An artificial electron acceptor, such as phenazine methosulphate, caused an increase in $^{14}CO_2$ formation from glucose-1-^{14}C, and McLean suggested that the increase in glucose oxidation by the pentose phosphate pathway is mediated by the rate of reoxidation of TPNH; this can be done either by an electron acceptor or by insulin which stimulates a process of reductive synthesis like, for example, the synthesis of long chain fatty acids. Two papers giving additional evidence for the concept that insulin has an action which is independent of carbohydrate metabolism are those of Kostyo (301) and of Wool & Krahl (302). Kostyo has shown a very significant insulin effect, *in vitro*, on the incorporation of leucine-2-^{14}C in the protein of diaphragm muscle from hypophysectomized rats. The presence of glucose in the medium did not influence the incorporation of leucine either in the absence or presence of insulin. Wool & Krahl have also demonstrated an insulin effect on the incorporation of amino acids into protein in conformity with results previously obtained by other authors and have devised an experimental technique which showed that the insulin effect was independent of the transport of amino acids into the cells. The relationship between glucose oxidation and fatty acids, and cholesterol synthesis (303) and protein synthesis (304), which has been recently demonstrated to depend largely on the generation of reduced pyridine nucleotide, particularly TPN, is of interest in relation to the suggested influence of insulin on the TPN/TPNH system and the known effects of this hormone on fat and protein synthesis.

Randle & Smith (112, 305) have reported that anaerobiosis, 2,4-dinitrophenol, sodium arsenite, sodium arsenate, and sodium salicylate increased glucose uptake and the release of potassium by the isolated diaphragm. Since the lack of oxygen and the cell poisons studied have the common property of inhibiting oxidative phosphorylation, they advanced the hypothesis that glucose entry into the muscle cell was restrained under normal conditions by a process dependent on a supply of a substance generated during oxidative phosphorylation and that insulin may promote glucose uptake by preventing access of this substance to the process regulating glucose entry. The problem of insulin action on sugar permeability in rat diaphragm muscle has been studied by Norman *et al.* (306) who followed the penetration of non-utilizable sugars (using chemical and radioactive measurements) in preparations of cut hemidiaphragms and in the so-called "cage preparations" of Kipnis & Cori (307) in which all the muscle fibers are intact. They concluded from their findings that, in preparations where muscle fibers were intact, there was an equilibration of the sugars in a small fraction of the total cell water and that this fraction was increased by insulin. In preparations with cut fibers, however, where the entire cell water was available for sugar penetration, insulin had an accelerating effect on the rate of equilibration, which they suggested

could be accounted for by an effect on the transfer through the remaining part of the intact membrane or on the cytoplasm to facilitate intracellular diffusion. However, as insulin had a specific effect on glycogen synthesis in cut hemidiaphragms that cannot be accounted for by an increase in glucose uptake, the authors concluded, in accordance with the results of Beloff-Chain and co-workers (308), that the action of insulin cannot be solely to modify the barrier qualities of the cell membrane but could have an effect on a specific diffusion pathway for glucose (or a glucose metabolite) between the membrane and the regions in the sarcoplasm where the enzymes concerned with glycogen synthesis are localized.

Barrnett & Ball (309) have investigated the problem of the mode of action of insulin with a new approach. They have shown for the first time, using the electron microscope, that morphological changes in rat adipose tissue accompanied the metabolic changes produced by insulin. From the results, they tentatively suggested that one of the actions of insulin is to stimulate pinocytosis, resulting in alterations of the cell membrane permeability.

Wick *et al.* (310) have studied the effect of insulin on the uptake of 6-deoxy-6-fluoroglucose in isolated rat diaphragm muscle and in the eviscerated nephrectomized animal. The authors concluded that the inability of insulin to alter the rate of cell entry of 6-deoxy-6-fluoroglucose, a compound with the same stereo-configuration as glucose but incapable of being phosphorylated by hexokinase, indicated that insulin action was in some way associated with the hexokinase reaction in accelerating the cell entry of sugars. However, the structure of this sugar, although having the same stereo-configuration as glucose, is such that it cannot participate in the metabolic reactions known to be stimulated by insulin; hence, the results of these experiments do not necessarily imply that hexokinase is implicated. Kipnis & Cori (311), in a study on the penetration and phosphorylation of 2-deoxy-glucose in the rat diaphragm, have shown that the phosphorylation and penetration of this substance ceased when the internal concentration of 2-deoxy-glucose-6-phosphate was 0.02 *M*, but, in the presence of insulin, the penetration and phosphorylation continued until the concentration was 0.05 *M*. The authors suggested that insulin either relieved the inhibition of 2-deoxyglucose penetration by 2-deoxy-glucose-6-phosphate or allowed the latter to occupy a larger cell volume analogous to its effect on free sugars. Holt *et al.* (199) have shown that the cornea and lens of bovine eyes oxidized glucose almost entirely via the pentose shunt and that the oxidation was not influenced by insulin. In the retina, however, both the Embden-Meyerhof pathway and the pentose shunt appeared to function, and they were both stimulated by insulin.

Bhattacharya (312), who has studied the influence of ions on glucose uptake and on the effect of insulin in the isolated rat diaphragm, has suggested that two distinct processes are involved in glucose uptake, one of which is dependent on insulin and the other independent. Ungar & Kadis (313) have reported an effect of insulin on the total sulfhydryl groups of diaphragm

muscle as measured by an amperometric titration method. In agreement with the findings of other authors, they showed that insulin increased the ratio K:Na ions, and, furthermore, they found that it decreased the sulfhydryl groups by about 22 per cent. Serum from guinea pigs sensitized to purified bovine insulin has been shown by Wright (314) to inhibit the stimulation of glucose uptake by insulin in the isolated rat diaphragm. Krahl, Tidball & Bregman (315) have described a procedure for the preparation from serum (from both normal and diabetic rats) of fractions of β-lipoprotein nature that are uniformally inhibitory to glucose uptake by rat diaphragm muscle. Rafaelsen (316) has described a technique of isolating rat spinal cord for experiments *in vitro*. With this preparation, he found that the glucose uptake was increased by about 50 per cent when insulin was added to the incubation medium. Glucose uptake by spinal cord tissue from alloxan diabetic rats was about 25 per cent lower than that found in normal rats. Ball, Martin & Cooper (317) have utilized the finding of Winegrad & Renold (318) that $^{14}CO_2$ formation from glucose was increased by insulin in rat adipose tissue to devise a simple method for measuring the insulin effect on glucose utilization in adipose tissue; the total gas pressure changes were measured in Warburg vessels in the absence of a CO_2 absorbent. Oyama & Grant (319) have developed a method of estimating the effect of insulin *in vitro* on glucose uptake by using pooled mice hemidiaphragms. The authors claimed that this technique is preferable to that generally employed, using rat diaphragm muscle, both in terms of sensitivity and reproducibility.

The precise role of insulin in the regulation of hepatic metabolism is still a controversial problem, which claims the attention of a number of investigators. Considerable evidence has accumulated that insulin has no direct effect on carbohydrate metabolism in the liver, but a number of authors still hold the opposite view. Reichard and co-workers (320) have carried out experiments in which "trace" doses of ^{14}C-labelled glucose were injected into dogs, and the blood sugar content and specific activity were measured at different time intervals. Under normal conditions, there is an exponential drop in the specific activity of the blood glucose caused by a constant replacement of blood glucose with new glucose molecules. The authors found that, when insulin was administered to the animals, there was a "plateauing" of the blood specific activity which was interpreted as an indication of a direct suppressive action of insulin on hepatic glucose output; other possible interpretations of the findings are discussed. Shoemaker, Mahler & Ashmore (321) and Shoemaker *et al.* (322) have also studied hepatic glucose production in response to insulin in dogs by using a technique involving direct simultaneous measurements of concentration gradients across the liver (total splanchnic and non-hepatic splanchnic areas) combined with a measurement of hepatic plasma flow. They found no direct effect of insulin (0.1 to 2.0 units/kg.) on hepatic glucose output in the immediate period following insulin injection; an increased hepatic glucose output was observed in the period of recovery from hyperglycemia. In this dose range, an insulin effect on glucose uptake was observed in the non-hepatic splanchnic area. The lack of a

direct insulin effect on hepatic glucose output has been corroborated by liver perfusion experiments (322) in agreement with the findings of Beloff-Chain and co-authors (323).

Experiments on dogs described by de Bodo *et al.* (324), in which trace amounts of ^{14}C-glucose were given as a priming injection and the radioactivity of the blood was maintained constant by a continuous infusion of trace amounts of ^{14}C-glucose, provided further evidence that the hypoglycemia produced by insulin was almost entirely caused by an increased glucose uptake by the tissues.

Diabetes.—A number of studies on alterations in enzyme activities and in concentrations of some intermediates of carbohydrate metabolism in tissues of experimental diabetic animals have been reported. Although some of these findings are of interest, they have not led to a clarification of the initial block in carbohydrate metabolism that occurs in diabetes. It is obviously difficult in this type of experiment to differentiate between alterations caused directly by a deficiency of insulin, i.e., by the diabetic state, and those which are secondary effects. Shaw & Tapley (325), using a sensitive colorimetric technique, have measured oxalacetate content of livers from normal and alloxan diabetic rats. They found that in the livers of diabetic animals, in which the acetoacetate content was shown to be very high as compared with the controls, the oxalacetate content was normal. This finding, the authors observed, may explain why succinate and other precursors of oxalacetate fail to relieve ketosis in diabetes. Harper (326) has reported a rise in liver glucose-6-phosphatase activity in alloxan diabetic rats and in rats which had been starved for 24 hr. This increase in glucose-6-phosphatase activity could account, at least in part, for the finding of Steiner & Williams (327) that the glucose-6-phosphate content of livers of fed normal rats was five to six times greater than that of fasted normal rats and about four times that of fed diabetic rats. As insulin treatment did not increase the glucose-6-phosphate pool of the liver during the period in which it increased fatty acid synthesis, glycogen synthesis, and the hexose monophosphate shunt in the liver of alloxan diabetic animals, the deficiency in glucose-6-phosphate is probably only a subsidiary factor in accounting for the decreased activity of the hexosephosphate shunt known to occur in diabetes [Bloom (328)].

Segal & Washko (329) have carried out a study to determine if the increased glucose-6-phosphatase activity observed in diabetes was caused by an increased quantity of the enzyme or if the properties of the enzyme altered in the direction of an increased catabolic potency or if both factors participated in the effect. The elevated Michaelis constant of the enzyme obtained with diabetic rat liver homogenates persisted in isolated washed microsome preparations, but, upon disruption of the microsomes, the Michaelis constants of the liver enzymes from diabetic and normal rats became indistinguishable. The authors discussed possible interpretations of these findings and concluded that a modification in the structure or composition of the microsomes themselves accompanied, and perhaps underlay, the altered kinetics and activities of glucose-6-phosphatase in the diabetic state. Siper-

stein & Fagan (330) have studied glucose oxidation and lipogenesis in diabetic rat liver to see if the reduction in fatty acid synthesis that occurs in the diabetic state could be correlated with a deficiency in one pathway of glucose oxidation. They found that both the Embden-Meyerhof pathway and the pentose phosphate pathway of glucose oxidation were decreased in diabetes, but that the decreased lipogenesis was attributable to the decrease of the pentose phosphate pathway, which produced a deficiency in TPNH. The use of a TPNH-generating system, such as isocitrate plus TPN, restored lipogenesis. Lamprecht & Trautschold (331) have reported that, when a high concentration of glucose is injected into an alloxan diabetic rat, an accumulation of triose phosphate occurs in the liver and this effect was counteracted by a large dose of insulin. The authors explained this effect as caused by an inhibition of triose phosphate dehydrogenase in diabetic liver. Beatty *et al.* (332) have observed a decrease in creatine phosphate, total acid soluble phosphate, and total phosphate in muscle from severely alloxan diabetic rats and an increase in the trichloracetic acid extractable glycogen. The authors suggested that the decrease in creatine phosphate, although only about 7 per cent, may be of importance as, if it occurred throughout the muscle, the total overall drop in available energy would be considerable. Frunder, Richter & Börnig (333) and Richter, Frunder & Börnig (334) have reported that, in alloxan diabetic mice, the glucose, raffinose, and inulin free space volumes were considerably reduced and were increased by insulin. It has been postulated by some authors that the decrease in the peripheral utilization of ketones in the diabetic animal may be a contributory factor to diabetic ketosis. In this connection, Beatty *et al.* (335) have studied acetoacetate and glucose uptake in diaphragm muscle and in preparations of skeletal muscle fibers; they found that the uptake of glucose and acetoacetate was reduced in both types of muscle of alloxan diabetic rats as compared with the muscle of normal rats. Tessari (336) has reported that cartilage from alloxan diabetic rats showed a decreased ability to take up glucosamine *in vitro* and that the addition of insulin to the incubation medium restored the uptake to normal. As one of the end products of glucosamine metabolism is N-acetyl-glucosamine, a major component of the mucopolysaccharides and, thus, important in the calcification of the organic bone matrix; a deficiency of glucosamine uptake by cartilage might explain the abnormal bone-forming process which is sometimes observed during diabetes.

The mode of action of the oral antidiabetic drugs is still being widely investigated. A monograph on the hypoglycemic arylsulphonamides, including articles on pharmacological, metabolic, and clinico metabolic studies, has been published (337). There is considerable evidence that the hypoglycemic effect of these drugs is mediated through a stimulation of the secretion of endogenous insulin. This conclusion is based on data which indicate that, in the absence of functional pancreatic β-cells, these compounds are inactive. Objections to this conclusion are based on the differences which have been observed between the effect of the sulphonylureas and of insulin on carbohydrate metabolism. In this connection, Schambye & Tarding (338) have

shown (in agreement with the findings of other investigators) that tolbutamide has an inhibitory effect on hepatic glucose output, whereas they believe that the hypoglycemic effect of insulin is almost entirely attributable to a stimulation of glucose utilization by peripheral tissue. The question as to whether or not insulin has a direct effect on hepatic glucose output has been discussed above (see p. 332). Madison *et al.* (339) have given evidence that, when insulin is administered under certain experimental conditions it causes a decrease in hepatic glucose output, and they, therefore, concluded that this action of the arylsulphonamides could also be explained by a stimulation of the endogenous secretion of insulin. Evidence from experiments, *in vitro*, has shown that the direct effect of the arylsulphonamides on isolated tissue is very different from that of insulin. As has been discussed above, insulin increases glucose utilization by stimulating exclusively the synthetic reactions of the cell; the arylsulphonamides, on the other hand, have been shown to interfere with the synthetic reactions and to stimulate glucose uptake only by accelerating catabolic reactions. Thus, Renold *et al.* (340) have shown that tolbutamide and chloropropamide inhibited ketogenesis by liver slices and inhibited lipogenesis in rat adipose tissue. In the latter tissue, the oxidation of glucose to CO_2 was stimulated mainly by the phosphogluconate pathway. Freinkel & Ingbar (341) have also reported an inhibition by tolbutamide (orinase) of lipid synthesis from glucose in the isolated rabbit kidney cortex. In addition to the adipose tissue, the main site of insulin action is in the muscle; most investigators have not been able to observe an effect of the arylsulphonamides in the isolated muscle (342), although Lundbaek, Nielsen & Rafaelsen (343) have reported an effect of these drugs on glucose uptake in isolated rat diaphragm under certain experimental conditions but showed that these drugs, unlike insulin, did not effect glycogen synthesis.

Another group of compounds which has been known for many years to have hypoglycemic properties is the biguanides. In a systematic survey, Ungar, Freedman & Shapiro (344) have shown that phenethylbiguanide caused severe hypoglycemia in several species. As the drug was shown to have an effect in alloxan diabetic animals, it was concluded that its action is not dependent on the presence of insulin. It appears probable that the hypoglycemia produced by this drug is caused by an increased peripheral uptake of glucose. It was, in fact, shown by Williams *et al.* (345) that phenethylbiguanide stimulated glucose uptake *in vitro* in rat diaphragm muscle [this result was also obtained by Rafaelsen (346)], but, in contradistinction to insulin, it decreased the glycogen storage and increased lactic acid production. Forbath & Clarke (347) have confirmed these findings.

Adrenal hormones.—The effect of epinephrine on phosphorylase activity has been studied extensively by Sutherland and his colleagues in recent years, and the fundamental observations of this group of investigators has been discussed by Perske, Kvam & Parks (348). The latter authors have measured the relative activities of the various rate-limiting enzymes of hepatic carbohydrate metabolism in rat liver and, on the basis of their data, have discussed the function of hepatic phosphorylase activation in epinephrine hypergly-

cemia. They pointed out, firstly, that, as the enzyme is readily reversible, its activation could only accelerate the attainment of an equilibrium, an equilibrium which, in fact, favors glycogen deposition; secondly, in normal rats it was found that the maximal hepatic phosphorylase activity in the direction of glucose-1-phosphate approached that of glucose-6-phosphatase, and, therefore, it was unlikely that phosphorylase activity was a rate-limiting step in the glycogen to glucose pathway. Furthermore, the authors were unable to demonstrate a change in phosphorylase activity during epinephrine-induced hyperglycemia. The authors, therefore, suggested that the hormone must act by altering, in some way, the equilibrium conditions of the phosphorylase reaction or by activating an alternative pathway for glycogen breakdown.

Sokal & Sarcione (349) have made a detailed study of the effect of epinephrine on muscle and liver. They concluded from their results that, to produce glycogenolysis in the liver, five to ten times as much epinehprine was required as that which was effective in the muscle. The glycogenolytic effect of epinephrine in the isolated perfused rat liver has been demonstrated by Sokal, Miller & Sarcione (350). It has been generally assumed that, when epinephrine is administered to animals, the lactic acid produced from muscle glycogen is converted into glucose and glycogen by the liver. However, when ^{14}C-labelled lactate was injected into rabbits that had received a dose of epihephrine, Drury & Wick (351) found that the extra hepatic tissues readily oxidized the lactate to CO_2, and, in fact, the $^{14}CO_2$ formation from ^{14}C-lactate was much more rapid than from ^{14}C-glucose. The rapid decrease in the specific activity of the circulating ^{14}C-lactate showed that following epinephrine treatment new lactate was added to the blood at a high rate. The authors concluded that the circulating lactate produced by epinephrine contributed a large part of the energy needs of the body. Dickman, Wiest & Eik-Nes (352) have studied the effect of epinephrine on the metabolism, *in vivo*, of glucose in normal dogs, by injecting tracer doses of ^{14}C-glucose to animals receiving an infusion of non-labelled glucose with and without the concomitant administration of epinephrine. The results demonstrated that epinephrine caused an increase in the total concentration of blood glucose, a decrease in the removal of ^{14}C from the blood, an appearance of $^{14}CO_2$, a retardation in the rate of decrease of the blood glucose specific activity, and an increase in the level of blood lactic acid. These results are in agreement with the generally accepted theory that epinephrine has a dual function: firstly, to increase glucose output by the liver, probably by glycogenesis, and, secondly, to decrease the uptake of circulating blood glucose by the tissue.

Altszuler and his colleagues (353, 354) made detailed studies on the influence of adrenalectomy on carbohydrate metabolism in the dog. Trace amounts of ^{14}C-glucose were given as a "priming injection" to unaesthetized animals followed by a continuous constant infusion of glucose. They showed that, in the adrenalectomized dog in the postabsorptive state, the glucose pool and glucose turnover were only slightly lower than in normal animals and that glucose oxidation to CO_2 also showed no significant difference. This

finding is consistent with earlier observations, *in vitro*, which have shown that, both in rat liver slices (355) and the isolated diaphragm muscle of the rat (356), no very significant changes in carbohydrate metabolism were produced by adrenalectomy. These authors had previously shown that, in the hypophysectomized dog, there was a considerably decreased glucose pool and glucose turnover, which was increased when the adrenal steroids were administered. They suggested that these differences in the adrenalectomized dog, as compared with the hypophysectomized dog, both of which were deficient in adrenal steroids, can be attributed to the endogenous growth hormone in the adrenalectomized animals which contributed to the maintenance of a nearly normal glucose pool and turnover rate. The authors suggested, however, that this compensating effect of the growth hormone is, in some cases, insufficient to withstand the effect of fasting or the hypoglycemic effect of large doses of insulin in the adrenalectomized dog, and, therefore, the adrenal steroids probably also contribute to the maintenance of a normal glucose pool and glucose turnover rate.

Pituitary hormones.—Altszuler and co-authors (357) have studied the effect of growth hormone on carbohydrate metabolism in normal and hypophysectomized dogs by using the technique referred to above in which a primary injection of trace amounts of ^{14}C glucose was injected into dogs and a constant continuous infusion of glucose administered. Growth hormone increased the plasma glucose concentration, the body glucose pool, and the rate of glucose production and utilization in normal and hypophysectomized dogs. The source of the additional glucose produced is not known. The outstanding characteristic of the growth hormone regimen was the increase in glucose utilization. The authors discussed the effect in relation to the results obtained in experiments with the isolated rat diaphragm and proposed the following hypothesis to explain the discrepancy between *in vivo* and *in vitro* results. Growth hormone *in vitro* inhibited glucose uptake in muscle, but, in the muscle cell *in situ*, this inhibition was overcome because of an increased endogenous insulin secretion stimulated by the extra glucose produced by the liver in the growth hormone-treated animals. The hypersensitivity of the hypophysectomized dog to insulin manifests itself by a greater increase in the rate of glucose disappearance from the plasma and a failure to evoke an adequate stimulation of glucose formation in response to the hypoglycemia. Growth hormone treatment of hypophysectomized dogs, on the other hand, produced a decreased response to insulin. Using a ^{14}C-glucose dilution technique, Altszuler *et al.* (358) have shown that this decreased response to insulin was caused by a decrease in the rate of plasma glucose disappearance and by the effect of growth hormone to restore, in the hypophysectomized animal, its ability to form glucose in response to a hypoglycemic stimulus. The authors discussed possible interpretation of these findings but concluded that the mechanism of the antiinsulin effect of growth hormone is not clear. These results on the influence of growth hormone and hypophysectomy on insulin action are consistent with the findings recently reported by Manchester, Randle & Young (359). They found, in agreement with previous work, that

the basal glucose uptake of rat diaphragm muscle *in vitro* rises after hypophysectomy and falls after injection of growth hormone. Furthermore, they showed that, when diaphragm muscle was incubated in the presence of a high concentration of insulin (200 mμ/ml.), neither hypophysectomy nor treatment of rats with growth hormone had a significant effect on the glucose uptake of the isolated diaphragm. On the other hand, when the amount of insulin present was expected to be a limiting factor in glucose uptake (i.e. at an insulin concentration of 0.1–1 mμ/ml.), the greatest response to insulin was seen with diaphragm from hypophysectomized rats and the smallest response with muscle from rats treated with growth hormone. The authors concluded from this finding that growth hormone affected the sensitivity of the system to insulin.

An *in vitro* effect of growth hormone has recently been demonstrated in adipose tissue by Winegrad *et al.* (360). They have shown that, in this tissue, growth hormone, like insulin, stimulated the oxidation of glucose by adipose tissues from normal or alloxan diabetic rats. In contrast to insulin, however, growth hormone did not stimulate the synthesis of long chain fatty acids from glucose, nor did it correct the defect in fatty acid synthesis in adipose tissue from alloxan diabetic rats. Furthermore, although the use of glucose-1-^{14}C and glucose-6-^{14}C showed that insulin stimulated glucose oxidation via the phosphogluconate pathway, growth hormone stimulated the oxidation of both C-1 and C-6 but had a more marked effect on the latter. Cristophe & Mayer (361) have confirmed previous results showing that a single injection of growth hormone to fasted rats produced a hypoglycemic response which they indicated was attributable to increased glucose utilization. They were of the opinion that this effect was not caused by increased insulin secretion because the action of insulin would be immediate, whereas the effect was observed only after 2 hr. Furthermore, repeated injections of growth hormone decreased the magnitude of the response. Harper & Young (362) have reported that hypophysectomy causes a fall in glucose-6-phosphatase activity in liver which is counteracted by treating the hypophysectomized animals with cortisol or thyroxine.

Steroid hormones.—There is some evidence that the steroid hormones can influence certain aspects of carbohydrate metabolism, but work in this field is still only of a very preliminary nature. Pesch & Topper (363) have shown that certain steroid hormones markedly stimulated the formation of $^{14}CO_2$ from ^{14}C-labelled galactose incubated with liver slices. The site of stimulation has not yet been found. Sobel & Eckstein (364) have found that, in the female rat, the activity of succinic dehydrogenase increased following ovariectomy and that the gonadal hormones inhibited to varying extents this effect. Conchie & Findlay (365) have shown that sex hormones may influence the activity of certain glycosidases in the rat and mouse. It is known that estrogen acts specifically on increasing glycogen storage in rat uterus, whereas posterior pituitary extracts, oxytocin, and adrenal medullary hormones rapidly deplete the uterine glycogen store. Leonard (366) has correlated the effect of these hormones on glycogen with an effect on the uterine phosphory-

lase in rats. McKerns *et al.* (367) have found that certain estrogenic substances administered to rats for a short period, *in vivo*, stimulated glucose utilization in the isolated diaphragm muscle. Furthermore, the estrogen treatment was shown to have a potentiating effect on insulin action in muscle. Thus, in diaphragm from ethinyl estradiol-treated animals, the insulin effect on glucose uptake in the muscle was often increased two to three times; the effect of insulin on glycogen deposition was increased to a lesser extent. The mechanism of the potentiating effect of estrogen on insulin is discussed in relationship to the inhibition of adrenal corticoid secretion observed in estrogen-treated rats.

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METABOLISM OF SULFUR COMPOUNDS[1,2] (SULFATE METABOLISM)

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So much work has been published on sulfur compounds in the fourteen years since the last review in this series [Lugg (1)] that we have not attempted to cover the whole field. This paper is regretfully restricted to the metabolism of sulfate, and many other areas must be slighted in which valuable developments have appeared. However, a few outstanding reviews and general works dealing with particular sulfur compounds should be mentioned: methionine (2), glutathione (3), lipoic acid (4), biotin and other compounds (5), protein sulfhydryl groups (6, 7), and a collection of papers in memory of C. Fromageot, including his complete bibliography (8). General reviews on sulfur metabolism have been published by Bersin (9), Young & Maw (10), and Dziewiatkowski (11).

Although many good whole animal studies have appeared on the incorporation and distribution of sulfate [Dziewiatkowski (12)] and on the conjugation of phenolic compounds with sulfate, we shall report mainly enzymological work. Recent knowledge of enzyme reactions involving sulfate has been reviewed by Lipmann (13). The activation of sulfate to form a "high-energy" intermediate is the first step in its assimilation. Two paths are then open: the sulfate may be reduced, or it may be transferred, in which case the reaction is catalyzed by sulfokinases and various esters are formed. And finally, there are hydrolytic enzymes, the sulfatases, which split almost all sulfate esters.

Sulfate Activation

DeMeio and his co-workers were the first to study phenyl sulfate formation in a homogenate (14). They showed that in a liver homogenate oxygen and mitochondria were necessary for phenyl sulfate synthesis and that ATP would replace these requirements (15). These workers were also among the first to show that the sulfate activating and -transferring systems could be separated (16). Bernstein & McGilvery (17) studied the conjugation of *m*-aminophenol in liver homogenates. By studying the kinetics of this system, they deduced that an intermediate activated sulfate was formed and that at least two enzymes were involved (18). They also showed that a compound accumulated that would rapidly transfer sulfate when ATP, sulfate,

[1] The survey of the literature pertaining to this review was concluded in September 1959.

[2] The following abbreviations are used in this review: AMP for adenosine monophosphate; APS for adenosine-5′-phosphosulfate; *p*-NP and *p*-NPS for *p*-nitrophenol and its sulfate, respectively; PAP for 3′-phosphoadenosine-5′-phosphate; PAPS for 3′-phosphoadenosine-5′-phosphosulfate; PP for pyrophosphate; TPN^+ and TPNH for oxidized and reduced triphosphopyridine nucleotide, respectively.

and Mg^{++} were incubated with their enzyme in the absence of a phenolic acceptor. Segal (19) extended the kinetic analysis of the system and studied the inhibition by iodoacetate in detail. He also reported that the enzyme preparation would form estrone sulfate from estrone, ATP, Mg^{++}, and inorganic sulfate.

Hilz & Lipmann (20) attempted to isolate the intermediate sulfuryl group carrier. They showed that incubation of ATP with radioactive sulfate and either *Neurospora sitophila* extract or a liver ammonium sulfate fraction led to the appearance of a labeled compound, which would transfer its sulfuryl group to *p*-nitrophenol. Since the stoichiometry of the reaction suggested that inorganic pyrophosphate was formed from ATP and since the compound had an absorption peak at 260 mμ, it appeared to be a sulfuryl adenylate. Beginning with these observations, Robbins & Lipmann (21, 22) isolated the compound by Dowex-1 chromatography. By a combination of chemical and enzymatic methods, "active sulfate" was characterized as 3′-phosphoadenosine-5′-phosphosulfate, or PAPS (Figure 1).

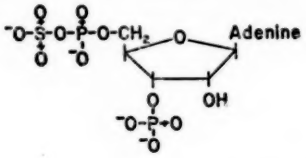


FIG. 1. Structure of 3′-phosphoadenosine-5′-phosphosulfate.

This structure has recently been confirmed by chemical synthesis by Baddiley *et al.* (23). The enzyme system that synthesizes PAPS has been found in a variety of plant, animal, and microbial sources, including liver and *Neurospora sitophila* (20), yeast (24), chick embryo (25), colonic mucosa (26), mast cell tumor (27), *Fusarium solani* (28), marine algae (29), and the marine snail *Busycon* (29). The isolation method for substrate amounts of PAPS has been improved recently by Brunngraber (30).

Following the characterization of PAPS, work on the mechanism of its enzymatic synthesis from ATP progressed rapidly. Bandurski *et al.* (31) and Robbins & Lipmann (32) showed that sulfate activation in yeast requires two enzymes. The first enzyme catalyzes the displacement of pyrophosphate from ATP with the formation of APS. This enzyme has been called ATP-sulfurylase. The second reaction, catalyzed by APS-kinase, is the phosphorylation of the 3′-hydroxyl group of APS by ATP to form PAPS:

$$\begin{array}{l} ATP + SO_4^{-2} \rightleftarrows APS + PP \\ APS + ATP \longrightarrow PAPS + ADP \\ \hline 2ATP + SO_4^{-2} \longrightarrow PAPS + ADP + PP \end{array} \qquad 1.$$

An additional enzyme, whose function remains obscure, was found in yeast (32, 33); it catalyzes the following reaction: $APS + P \rightarrow ADP + SO_4^{-2}$. This

enzyme has been called ADP-sulfurylase, although the reaction between ADP and sulfate has not been observed. Synthetic APS, prepared by Baddiley *et al.* (34) and Reichard & Ringertz (35), is identical with the enzymatic material and has been used as a substrate for the study of these reactions.

The ATP-sulfurylase of yeast is a stable acidic protein which has been purified extensively by Robbins & Lipmann (33, 36) and Wilson & Bandurski (37). The outstanding characteristic of the sulfurylase reaction is the extremely unfavorable equilibrium for APS formation. At pH 8 and 37°, Robbins & Lipmann (36) found an apparent equilibrium constant of about 10^{-8}.

$$K_{app} = \frac{[APS] \times [PP]}{[ATP] \times [SO_4]} = 10^{-8} \qquad 2.$$

Wilson & Bandurski (37) obtained a value of 4×10^{-8} at pH 7.5 and 30°. Since the equilibrium of the sulfurylase reaction is so unfavorable, the forward reaction proceeds to a reasonable extent only when both products, PP and APS, are removed by subsequent reactions. Pyrophosphate is removed by hydrolysis and APS by phosphorylation in the APS-kinase reaction, both of these reactions being strongly exergonic. APS-kinase also relieves the severe kinetic inhibition of the ATP-sulfurylase reaction by APS.

ATP-sulfurylase is completely specific for ATP but will use a number of inorganic anions in place of sulfate (37). With all of these anions, of which molybdate is the most active, an unstable anhydride that hydrolyzes spontaneously is presumably formed between AMP and the anion. The over-all reaction then observed is a splitting of ATP to AMP and PP. The APS-kinase reaction has not been studied as extensively as sulfurylase, but the expected stoichiometry has been shown. The enzyme has a very high affinity for APS (33).

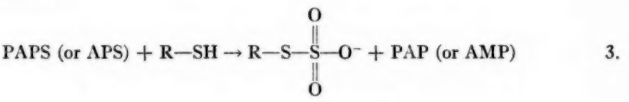
The fact that ATP-sulfurylase has been found in liver homogenates (36, 38) is evidence that the enzymatic mechanisms of PAPS formation are the same in liver as in yeast.

Sulfate Reduction

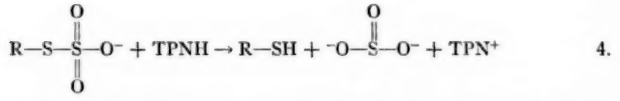
Reduction of sulfate to sulfite.—One of the most important steps in the utilization of sulfate in plant and bacterial systems is the reduction of sulfate to sulfite. Thermodynamically, this is an extremely unfavorable process. The reaction $SO_4^{-2} + H_2 \rightarrow SO_3^{-2} + H_2O$ has a standard free energy change of +14 kcal. (39). As an analogy with carboxyl group reductions in enzymatic systems, it is interesting to find that sulfate reduction also requires an activation prior to the reduction step. Chemically, this is understandable since esters and anhydrides, in general, are more readily reduced than the corresponding anions.

Two enzymatic systems that reduce sulfate have been described. Wilson & Bandurski (40) and Hilz & Kittler (41) have studied the reducing system in bakers' yeast. The principle requirements for either sulfite or sulfide formation appear to be ATP, Mg^{++}, and TPNH. ATP and Mg^{++} are probably

needed only for the activation process. Wilson & Bandurski (40) show that PAPS can be reduced by TPNH, and Hilz & Kittler (41) find that ATP can be replaced by *p*-NPS plus PAP in the presence of phenol sulfokinase [see section on sulfokinases; also (42)]. Hilz & Kittler (41) make the interesting suggestion that a substituted thiosulfate may be an intermediate in the reduction process.

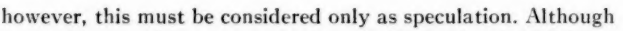


$$\text{PAPS (or APS)} + \text{R—SH} \rightarrow \text{R—S—}\overset{\overset{\text{O}}{\|}}{\underset{\underset{\text{O}}{\|}}{\text{S}}}\text{—O}^- + \text{PAP (or AMP)} \qquad 3.$$

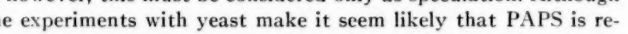


$$\text{R—S—}\overset{\overset{\text{O}}{\|}}{\underset{\underset{\text{O}}{\|}}{\text{S}}}\text{—O}^- + \text{TPNH} \rightarrow \text{R—SH} + {}^-\text{O—}\overset{\overset{\text{O}}{\|}}{\underset{\underset{\text{O}}{\|}}{\text{S}}}\text{—O}^- + \text{TPN}^+ \qquad 4.$$

At present, however, this must be considered only as speculation. Although many of the experiments with yeast make it seem likely that PAPS is reduced, the possibility must also be considered that APS is the substrate for reduction. This is especially true in light of the *Desulfovibrio* system discussed below. Even in the case in which the generating system *p*-NPS plus PAP was used, APS might be formed by phosphatase action on PAPS in crude yeast preparations.



$$p\text{-NPS} + \text{PAP} \leftrightharpoons p\text{-NP} + \text{PAPS} \qquad 5.$$



$$\text{PAPS} \rightarrow \text{APS} + \text{P} \qquad 6.$$

A much more active sulfate-reducing preparation has recently been described by Peck (43) and by Ishimoto (44). This system can be extracted from *Desulfovibrio desulfuricans* and obviously has a different physiological function from the yeast enzyme. In yeast, sulfate reduction is presumably for the purpose of assimilation, while in *Desulfovibrio* sulfate serves as the terminal electron acceptor. It remains to be seen whether the details of the enzymatic mechanisms in the two systems are different. Peck (43) has investigated the *Desulfovibrio* system in detail and has shown that only sulfite is formed if the reduction is carried out at pH 7.75. At pH 6.0, hydrogen sulfide is the product. Sulfite formation from sulfate and hydrogen requires ATP, Mg^{++}, and cytochrome-c_3, and is stimulated about eightfold by the addition of methyl viologen. Methyl viologen presumably acts as an electron carrier between hydrogenase and the reductases present in the preparation. The extract contains an active ATP-sulfurylase, and the stoichiometry of the reduction reaction shows that for each mole of hydrogen taken up, one mole of ATP is split to AMP and pyrophosphate and one mole of sulfite is formed. Although no hydrogen is taken up in the absence of ATP, APS will substitute completely for ATP. This observation and the stoichiometry show clearly that APS and not PAPS is the substrate for reduction. The over-all system for sulfate reduction to sulfite in *Desulfovibrio* may be summarized as in Reaction 7.

$$\begin{aligned} &H_2 + \text{cyt.-}c_3(2Fe^{+++}) \rightarrow \text{cyt.-}c_3(2Fe^{++}) + 2H^+ \\ &ATP^{-4} + SO_4^{-2} + H^+ \rightarrow APS^{-2} + PP^{-3} \\ &APS^{-2} + \text{cyt.-}c_3(2Fe^{++}) \rightarrow AMP^{-2} + SO_3^{-2} + \text{cyt.-}c_3(2Fe^{+++}) \\ \hline &H_2 + ATP^{-4} + SO_4^{-2} \rightarrow AMP^{-2} + SO_3^{-2} + PP^{-3} + H^+ \end{aligned} \qquad 7.$$

Sulfite reduction.—After the initial reduction, subsequent steps in sulfate utilization are less clear. On the basis of the information available at present, a probable scheme for sulfate assimilation as it takes place in yeast and plants is as follows:

$$SO_4^{-2} \xrightarrow[\text{TPNH}]{\text{ATP}} SO_3^{-2} \xrightarrow{\text{TPNH}} H_2S \xrightarrow{\text{serine}} \text{cysteine} \qquad 8.$$

It is not likely that this series of reactions has any importance in mammalian or avian tissues.

Lezius (45) has studied an enzyme system in yeast that catalyzes the reduction of sulfite by TPNH; the product of the reaction is sulfide. No cofactors in addition to TPNH seem to be necessary. Since the preparation is rather unstable, it is not clear whether one or more enzymes are involved. Although the stoichiometry requires three moles of TPNH per mole of sulfite, it still may be possible that only a single enzyme is necessary.

$$SO_3^{-2} + 3\text{TPNH} + 5H^+ \rightarrow H_2S + 3\text{TPN}^+ + 3H_2O \qquad 9.$$

Sulfite reduction by *Desulfovibrio* has been studied with whole cell suspensions (46, 47) as well as with extracts. Ishimoto *et al.* (48) showed that very little reduction of sulfite occurred in cell-free systems unless a carrier such as methyl viologen was added. With the isolation and careful study of cytochrome-c_3 by Postgate (39), it became clear that this cytochrome is an important natural carrier in sulfite reduction as well as in sulfate reduction by *Desulfovibrio* (39, 49). From the free-energy data presented by Postgate, it is clear that sulfite reduction by reduced cytochrome-c_3 is an energy-yielding process. In view of the fact that the yeast system apparently uses TPNH, further investigations on the intermediate steps in *Desulfovibrio* will be of interest.

Thiosulfate will serve as a sulfur source for a number of microorganisms, in many of which systems have been found that reduce thiosulfate to an equimolar mixture of sulfite and hydrogen sulfide. Kawakami *et al.* (50) found that sulfide formation from thiosulfate in *Proteus vulgaris* requires a particulate fraction, a soluble enzyme, and a dialyzable cofactor. A similar system has been found in *Escherichia coli* by Artman (51). Postgate (47) was probably the first to recognize that, although thiosulfate is reduced rapidly, it is not on the main pathway of sulfate reduction. Thiosulfate reduction in *Desulfovibrio* has been studied in detail by Postgate (39) and by Ishimoto and his co-workers (48, 49, 52). When molecular hydrogen serves as the electron donor, cytochrome-c_3 functions as a major carrier, but methyl viologen is also active. In this respect the reaction is similar to sulfate and sulfite reduction.

The enzymatic mechanism involved in the reductive cleavage of thiosulfate has been made clear by the work of Kaji & McElroy (53) with a preparation from bakers' yeast. They showed that the enzyme merely accelerates the non-enzymatic reaction between thiosulfate and sulfhydryl compounds. They studied the reaction with glutathione, which is abundant in yeast, but found that cysteine and homocysteine were also active. Glucose-6-phosphate was able to serve as the ultimate electron donor by way of glucose-6-phosphate dehydrogenase and glutathione reductase. It perhaps deserves emphasis that, although thiosulfate reduction has been widely observed, this reaction is probably not involved in any way with the normal reactions of sulfate reduction and assimilation.

From the work of Schlossmann & Lynen (54), it appears that following reduction hydrogen sulfide is incorporated into cysteine by way of reaction with serine:

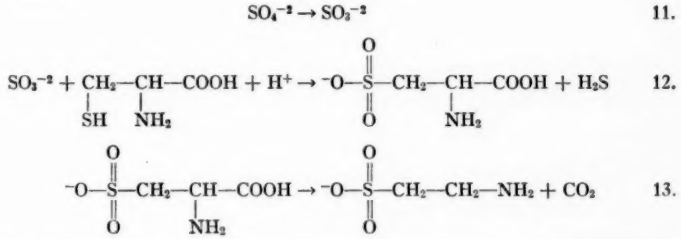
$$HOCH_2—CH(NH_2)—COOH + H_2S \rightarrow HSCH_2—CH(NH_2)—COOH + H_2O \qquad 10.$$

Presumably, then, methionine and the other reduced sulfur compounds in microorganisms are derived from cysteine, although the possibility of other condensation reactions with sulfide must also be considered. The most important problem in this connection is whether the sulfur of methionine is derived from cysteine or comes from a reaction between sulfide and homoserine. The condensation between hydrogen sulfide and serine is similar to the reaction between methyl mercaptan and serine in yeast described by Wolff & Black (55). Experiments by Fromageot & Perez-Milan (56) show clearly that sulfate is reduced by way of sulfite in intact higher plant tissues.

Sulfate reduction in animal tissues.—The incorporation of traces of S^{35}-sulfate into cystine has been noted in a number of animal species *in vivo* (57, 58, 59), but it has been pointed out that the conversion is undoubtedly caused by bacteria in the intestinal tract (57). In the hen, however, Machlin *et al.* (59) feel it is possible that the incorporation may not be caused completely by bacteria. The only reaction of any importance involving sulfate reduction in animal tissues seems to be the interesting synthesis of taurine, first noted by Boström & Åqvist (60), in liver following S^{35}-sulfate injection. The reaction is most striking in the chick embryo and has been studied there by a number of workers. Machlin *et al.* (61) found that, following the injection of a tracer dose of S^{35}-sulfate, over 65 per cent of the radioactivity was incorporated into taurine. Since no trace of radioactivity was found in cysteine or methionine, it was clear that taurine was derived directly from sulfate. Although the incorporation was extensive, it should be noted that taurine in the embryo was probably not all synthesized from sulfate, since the specific activity of taurine decreased rather rapidly after 12 days of incubation.

Lowe & Roberts (62) studied the products of sulfate fixation in chick embryo by paper chromatography and found detectable incorporation into taurine after 30 min. In the intact embryo, as well as in extracts, they found a number of compounds, one of which accumulated in considerable amount and appeared to act as a precursor of taurine. Chapeville & Fromageot (63)

studied the reaction of sulfate and sulfite in chick embryo extensively. They have provided convincing evidence that taurine is formed by the decarboxylation of cysteic acid. They propose the following scheme for sulfate fixation in the embryo:



Reaction 11, the reduction of sulfate to sulfite, takes place in the vitelline chamber. The reaction between sulfite and cysteine, or a compound derived from cysteine, to give cysteic acid and hydrogen sulfide occurs in the yolk. The specific cysteic acid decarboxylase seems to be present in both the yolk and embryo. The first two reactions are very interesting and rather surprising and deserve further careful study. It is especially surprising to find the reduction of sulfate to sulfite, since this has been considered to be a reaction occurring only in plants and microorganisms.

Sulfokinases

Active sulfate (PAPS) is the central point from which a wide variety of transferring reactions radiate, and the enzymes catalyzing these transfers may be called sulfokinases [Lipmann (13)]. The existence of many of these reactions has been inferred from the presence of sulfate conjugates in urine and secretions and from the study of detoxication mechanisms (64). Among those which have been observed *in vivo* or in tissue preparations are the sulfurylation of many kinds of phenols (11, 65, 66, 67), including triiodothyronine (68) and tyrosine derivatives (69, 70); of arylamines (71); steroids (72, 73); mucopolysaccharides (11, 74, 75), heparin (76), and charonin (77); cerebrosides (78); and bilirubin (79).

A most important recent observation by Vestermark & Boström (80, 81) is that a number of aliphatic alcohols and polyhydroxy compounds can be conjugated as sulfates in particle-free extracts of rat liver. Aliphatic sulfates have also been detected in urine of rats fed alcohols ranging from methanol to amyl alcohol (82). The sulfates were not isolated but were well characterized by paper electrophoresis and chromatography, thus confirming the detoxication pathway that has been postulated for many years but never observed. It is not known at present what proportion of ingested alcohol may undergo this conjugation, nor are there studies as yet on the possible participation of PAPS as a sulfate donor.

Phenols.—Phenol sulfokinase of rabbit liver has been partially purified by Gregory & Lipmann (42); and with these preparations, where the possibility of more than one enzyme still exists, sulfate can be transferred from

PAPS to a variety of phenolic compounds. In the case of *p*-nitrophenol, the equilibrium was determined:

$$K_{app} = \frac{[p\text{-NPS}] \times [\text{PAP}]}{[p\text{-NP}] \times [\text{PAPS}]} = 26 \ (30°, \text{pH } 7.8) \qquad 14.$$

Thus, the ready reversibility of the transfer made possible the coupled reactions shown in Figure 2.

With the appearance of the color of *p*-nitrophenol as a measure of this reaction, an assay system sensitive to less than one millimicromole of PAP plus PAPS was developed. The same reaction was used for assay of the transferring enzyme and for testing acceptors other than phenol. Brunngraber (30) reported a variation of this scheme in which *m*-aminophenol was the acceptor and the *m*-aminophenyl sulfate was determined colorimetrically (17). The pH optimum here was lower than with phenol as acceptor, but this may concern the state of ionization of the reactants rather than the participation of different enzymes.

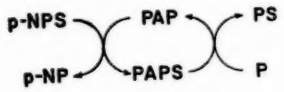


FIG. 2. Sulfate transfer scheme.

Although tyrosine-O-sulfate is normally found in human urine (83), tyrosine itself is not sulfated in the supernatant of a human or rat liver homogenate, according to Segal & Mologne (69). Derivatives of tyrosine, however, are sulfated if the carboxyl group is esterified or absent and the amino group is free. This may reflect a metabolic specificity in which tyrosine is sulfated only when bound in a peptide or protein [Blombäck *et al.* (70); Bettelheim (84); Dziewiatkowski & DiFerrante (85)]. Fourfold purification of the rat liver enzyme by ammonium sulfate fractionation (69) failed to separate the activity from *m*-aminophenol sulfokinase, and the same enzyme may be involved. So far there is no good evidence in favor of the existence of more than one broadly reactive phenol sulfokinase in any tissue examined.

Arylamines.—Some observations have been made by Boyland *et al.* (71) on the excretion of aromatic sulfamates in animal urine after administration of arylamines, and Kent & Pasternak (86) have found transfer activity toward arylamines in extracts of sheep colonic mucosa. On an enzymatic level Roy (87, 88) has studied preparations from rat or guinea pig liver, using 2-naphthylamine or aniline as acceptors. The sulfate donor was shown to be PAPS, but the arylamine sulfokinase was apparently different from phenol sulfokinase [Gregory & Lipmann (42)] in that it required Mg^{++} for maximal activity; it, too, is inhibited by sulfhydryl-blocking reagents.

In rat liver (88), the formation of 2-naphthyl sulfamate was stimulated by low concentrations of 17-ketosteroids, suggesting a more complicated pathway that might involve steroid sulfate as an intermediate. There is no recorded observation of any sulfamate as a naturally occurring metabolite, except for the case of heparin.

Choline.—Choline sulfate has been found in many molds and in some lichens and algae. It was first isolated from *Aspergillus sydowi* [Woolley & Peterson (89)]. This organism has recently provided extracts in which the enzymatic steps in the synthesis of choline sulfate could be studied. Kaji & McElroy (90) showed that radioactive sulfate was incorporated into the ester with ATP as energy source and postulated that PAPS might be an intermediate. The transfer from PAPS was confirmed by Kaji & Gregory (91), although the kinase was not extensively purified. A reducing agent such as cysteine was needed for maximal activity, as were magnesium ions. Attempts to show the reverse transfer, from choline sulfate to PAP, were unsuccessful, and there is no evidence at present that choline sulfate has a high enough group potential to serve as a storage form of active sulfate, a function suggested by Spencer & Harada (92), who investigated the distribution of choline sulfokinase among a number of microorganisms. The compound might serve, however, to store sulfur in the organism in a form utilizable in some other way. Choline sulfate, unlike most other naturally occurring sulfates, is not hydrolyzed by extracts of gastropod liver (which contain a variety of sulfatases) [Egami *et al.* (93)], and no choline sulfatase has been found in animal tissues or microorganisms [Egami & Itabashi (94)].

Steroids.—The excretion in mammals of steroid sulfates has long been known, and recently cell-free steroid-conjugating systems have become available for enzyme studies. DeMeio & Lewycka (95) first observed sulfation of dehydroepiandrosterone in a soluble extract of rat liver, and Segal (19) detected estrone sulfate in a similar system. Out of 32 steroids tested in a particle-free rabbit liver extract, Schneider & Lewbart (72) found that 14 were sulfated; further work has been done with rat liver fractions by Roy (96), in rat and beef liver by DeMeio *et al.* (97), and in frog liver homogenates by Bridgwater & Ryan (98). There is still considerable doubt as to how many separate sulfokinases there may be in the steroid field. A few conclusions can be drawn from the fractionation studies by Gregory & Nose (99) and Nose & Lipmann (100) on rabbit liver extract. Since preparative electrophoresis clearly shows that the sulfokinase for dehydroepiandrosterone is different from that for estrone (as an example of a steroid with a phenolic hydroxyl group), the phenolic and non-phenolic groups of steroids may be sulfated by two different enzymes, or, of course, the subdivisions may be more subtle. A partial separation of the activities toward estrone and *p*-nitrophenol was obtained, but this differentiation is less convincing, and the possibility still exists that all phenolic compounds are sulfated by the same enzyme.

In the case of non-phenolic steroids, certain of those with a 3-β hydroxyl group were the most readily sulfated, but this was done with an enzyme already fractionated for activity toward dehydroepiandrosterone, and other activities may reside in the original mixture. At any rate, sulfating activities toward dehydroepiandrosterone, androsterone, and pregnenolone were superimposable after electrophoresis. Some further conjectures on the relationship of structure to ease of sulfation are given by Schneider & Lewbart (72).

Mucopolysaccharides.—In the biosynthesis of mucopolysaccharides, two possibilities have been considered: (*a*) that a small sulfated intermediate is

polymerized; or (*b*) that a macromolecule is formed which is later sulfated. Recent evidence is in favor of the latter scheme. Suzuki & Strominger (101) found no incorporation of uridine diphosphoacetylgalactosamine sulfate into mucopolysaccharide in extracts of the isthmus region of hen oviduct, where the incorporation of sulfate from PAPS is known. The uridine compound is a potential intermediate normally present in this tissue (102). In addition, Korn (103) was unable to find any nucleotide-bound sugar sulfates in mouse mast cell tumor extracts, where such low molecular weight intermediates might exist if they were involved in the active synthesis of heparin, which is characteristic of the tissue.

Positive evidence for sulfate transfer to a polymer comes from studies of both heparin and chondroitin sulfate synthesis. The first indication that PAPS was a sulfate donor in the case of heparin was reported by Spolter & Marx (104) in homogenates of mouse mast cell tumor, and Korn (105) has reported the direct incorporation of sulfate from PAPS into the full carbohydrate chain of heparin in particle-free extracts of the tumor. It is not certain yet whether this sulfate is put into the normal sites in the molecule nor whether all the sulfate of heparin is thus put in, but the amide sulfate seems not to be involved in this case. Incorporation of radioactive sulfate into heparin in tumor slices, on the other hand, gives approximately equal distribution between ester and amide sulfate (106).

In the case of chondroitin sulfate, D'Abramo & Lipmann (25, 107) showed by tracer experiments that PAPS is a sulfate donor to mucopolysaccharide material in particle-free extracts of embryonic chick or beef cartilage. Delbrück & Lipmann (108) have recently isolated an enzyme system from extracts of calf embryo cartilage by chromatography on DEAE cellulose. The system transfers sulfate from PAPS to various polysaccharide acceptors, such as chondroitin sulfates A, B, and C and the corresponding chemically desulfated compounds, and also to heparitin and chondroitin sulfate from cornea. The optimum pH is 6.4, and the equilibrium probably strongly favors the sulfate polysaccharide, since no reversibility could be detected, even with sensitive methods. Adams (109), studying chick embryo extract, found that chondroitin sulfate C isolated from umbilical cord strongly supported sulfate incorporation, while chondroitin sulfate A from bovine trachea did not, and that chondromucoprotein from cartilage was inhibitory. Using the isthmus of hen oviduct, on the other hand, Suzuki & Strominger (101) demonstrated incorporation of sulfate with various primers or acceptors (chondroitin sulfates A, B, and C and "sulfated heptasaccharide"). In more detail (110), they showed that PAPS is the direct donor of sulfate and achieved a transfer of 64 per cent of its sulfate to chondroitin sulfate. Other acceptors were oligosaccharides from the degradation of chondroitin or chondroitin sulfate A, and also acetyl galactosamine and acetyl galactosamine sulfate, which were converted to the mono- and disulfate respectively. The enzymatically formed monosulfate was identical with that derived from uridine diphosphoacetylgalactosamine sulfate (101).

Some discussion [Spencer (111)] has been stimulated by the observation in Egami's laboratory (77) that sulfate can be incorporated from *p*-NPS into

charonin sulfate (glucan polysulfate) in acetone powder extracts of *Charonia lampas* mucous gland. A possibility is that this transfer reaction, being inhibited by phosphate and fluoride like some arylsulfatases, might be catalyzed by an arylsulfatase in analogy to the transfer reactions of phosphate catalyzed by phosphatase (112). It is hard to judge the biochemical significance of the transfer, since *p*-NPS is not known to be a normal metabolite.

Lipids.—There has been little detailed study of sulfate incorporation into brain sulfolipid [Holmgård (78)], but Goldberg & Delbrück (29) reported preliminary work on a particulate fraction of rat brain and on soluble fractions of rat liver extract that incorporate radioactive sulfate from PAPS into lipid products and are stimulated by the addition of N-acetyl sphingosine isomers. The reaction observed is complex, however, and other synthetic steps are probably involved since it is further stimulated by ATP and magnesium ions.

Sulfatases

Six types of sulfatases are usually recognized on the basis of the kind of sulfate ester they hydrolyze: arylsulfatases, steroid sulfatase, mucopolysaccharide sulfatases (chondrosulfatase and heparin sulfatase), glycosulfatase, myrosulfatase, and alkylsulfatase. Earlier work has been reviewed by Fromageot in 1938 (113), Soda in 1941 (114), Fromageot in 1950 (115), Dodgson in 1956 (116), and Dodgson & Spencer in 1957 (117). Methods for enzyme isolation and assay have been assembled by Fromageot (118) and Dodgson & Spencer (119). The function of the enzymes in the organism is at present unknown. Unless the substrates and functions attributed to them are not the true physiological ones, there seems to be little relationship between their distribution and the natural occurrence of their substrates (116).

Arylsulfatases.—The enzymes capable of hydrolyzing sulfate monoesters of phenolic hydroxyl groups exist in many types of organisms. They have been examined in greatest detail in microorganisms, molluscs, and mammals. Although they show a broad specificity with regard to the substituents of the phenolic ring, they are generally divisible into two classes [Dodgson *et al.* (120)]: Type I (inhibited by cyanide), which is more active toward *p*-NPS or *p*-acetylphenyl sulfate; and Type II (inhibited by sulfate or phosphate), for which the sulfate of nitrocatechol is a better substrate. In mammalian liver, the presence of three arylsulfatases has been established by Dodgson's group (120, 121); A and B are of Type II and are found in particle-free extracts, provided the mitochondria have been ruptured. Enzyme C (Type I) is associated with the microsomes and can be solubilized only with difficulty.

Arylsulfatase A has been purified extensively from ox liver by Roy (122), who first noted the presence of two sulfatases in the soluble fraction (123), and from human liver by Dodgson's group (124). Much effort has been expended in trying to analyze the anomalous reaction kinetics of this enzyme [Roy (123, 125); Baum & Dodgson (126); Andersen (127)]. The anomaly is shown by mammalian arylsulfatase A when acting on the sulfate of nitrocatechol or *p*-NPS, but not by arylsulfatase B, nor by the enzymes of *Alcali-*

genes metalcaligenes, *Patella vulgata*, *Helix pomatia*, or by taka-diastase [Baum *et al.* (124)]. It is manifested as a time-dependent loss of hydrolytic rate during incubation with substrate, followed by a partial recovery of the initial rate. The loss of activity depends on the presence of substrate, and together with many observations on the effects of activators and inhibitors, this has led Baum & Dodgson (126) to propose a rather involved mechanism. They postulate a slow reversible conversion of the enzyme when combined with substrate into a new form that is inhibited by higher concentrations of substrate or activated by combination with the reaction products (nitrocatechol or sulfate). Further attempts to analyze the system are being made by Andersen (127).

TABLE I

PROPERTIES OF ARYLSULFATASES

Sulfatase	Type	Inhibitors	Optimum pH	Substrate*	K_m	Reference
A	II	SO_4^{-2}, HPO_4^{-2}, F^-	double	NCS	8×10^{-4} *M*	(130)
B	II	SO_4^{-2}, HPO_4^{-2}, F^-	5.7	NCS	7×10^{-2} *M*	(130)
C	I	CN^-	8	*p*-NPS	2×10^{-3} *M*	(133)

* NCS for the sulfate of nitrocatechol (2-hydroxy-5-nitrophenol); *p*-NPS the sulfate of *p*-nitrophenol.

Because the involved kinetics of arylsulfatase A depend strongly upon the pH, the observed pH optima depend upon the time of incubation. For the human liver enzyme at short times with the sulfate of nitrocatechol as substrate, there are two, at pH 4.4 and 5.0 (124), which merge into one at longer times. Sulfate, phosphate, pyrophosphate, and fluoride are inhibitory, but cyanide is not. Sulfite is a strong competitive inhibitor for the ox liver enzyme, with 100 times the affinity of sulfate [Roy (122, 128)].

Arylsulfatase B, purified at least fifteenfold from human liver by Dodgson & Wynn (129), has simple kinetics; the corresponding enzyme from ox liver [Roy (130)] is not so straightforward but has not been studied in detail. The A and B enzymes can be separated by acetone fractionation [Roy (123)] or by paper electrophoresis [Dodgson *et al.* (131)]. Arylsulfatase B has a pattern of inhibition by anions similar to that of A and has a pH optimum of 6.1 for human liver (129). The optimum pH for ox liver is 5.7, and the affinity ($1/K_m$) for the usual substrate, nitrocatechol sulfate, is far less than that of the A enzyme (130).

There have been difficulties in assaying arylsulfatases A and B when they are present together, as in human urine. A solution to this has been worked out by Baum *et al.* (132) by utilizing a differential inhibition with chloride ions and other small differences in properties.

Arylsulfatase C is confined to the microsomal fraction of mammalian liver [Dodgson *et al.* (120, 131)]. The enzyme can be solubilized but only in the continuing presence of detergents [Roy (133); Dodgson *et al.* (134)] or by

treatment with crude pancreatic enzymes that give an unstable preparation [Dodgson *et al.* (135)]. Roy (133) has reported, for the ox liver enzyme, an optimum pH near 8 and a K_m at this pH of 2×10^{-3} for *p*-NPS, the standard substrate. Analysis of the dependence of K_m upon pH indicated a dissociating group in the enzyme with a pK of 7.8. The activity was little affected by chloride or sulfate and was sharply reduced by cyanide or sulfite.

These three mammalian arylsulfatases have been found in numerous human tissues [Dodgson *et al.* (131)] with the largest amounts in liver, kidney, and pancreas. Human serum and urine contain Type II enzymes, both A and B being present in urine [Dodgson & Spencer (136)]. The three enzymes are probably almost universally distributed in animal tissues, except that A seems to be lacking in lower vertebrates and C in the guinea pig, frog, and fish [Roy (137)]. The kinetics and distribution of hydrolytic activity toward 6-benzyl-2-naphthyl sulfate have been reported by Rutenberg & Seligman (138), who used homogenates of 17 tissues from each of seven mammalian species and a large number of human tumors. Unfortunately, the specificity of the three known sulfatases toward this substrate is not known.

Other sources of arylsulfatase are found in many types of organisms. An enzyme from a strain of *Alcaligenes metalcaligenes* isolated from tidal mud (139) has had considerable attention from Dodgson's group. Nitrocatechol sulfate and *p*-NPS are both split, and the pH optima are 8.0 and 8.75 respectively. With an enzyme purified 300-fold (140), an analysis of the dependence of K_m of various substrates upon pH gave evidence of two substrate-binding groups with pK of 8.2 and 9.4, but identification of the groups was not possible. There is strong inhibition by cyanide and none by fluoride, sulfate, or phosphate. Dodgson *et al.* (141) discuss at length the mechanism of hydrolysis and the effect of varying the substituents of the aryl group.

The sulfatase of *A. metalcaligenes* furnishes an example of a relatively rare type of inhibition (anti-competitive) in which the inhibitor (cyanide or hydrazine in this case) combines with the enzyme-substrate complex but not with the enzyme [Dodgson *et al.* (142)]. This enzyme also hydrolyzes tyrosine-O-sulfate, an unusual substrate as it is not split by mammalian arylsulfatase C, although theoretically it should be [Dodgson *et al.* (143)].

Marine molluscs have long furnished the most active arylsulfatase preparations [Soda (114)]. The enzymes from *Patella vulgata*, *Littorina littorea*, and *Helix pomatia* have more recently been studied by Dodgson *et al.* (144, 145), and the activity in other species has been recorded. These are inhibited by sulfate or phosphate ions, but they have not been extensively purified nor their specificities determined in detail. Taka-diastase (a preparation from *Aspergillus oryzae*) contains an enzyme which resembles Type I in that it is active toward *p*-NPS and inhibited by cyanide [Robinson *et al.* (146); Boyland *et al.* (147)].

The mechanism of arylsulfatase action has been determined by Spencer (148), who studied the products of liver and bacterial enzymatic hydrolysis of nitrocatechol sulfate or *p*-NPS in water containing O^{18}. In all cases, the isotope was found in the free sulfate, indicating a split of the O-S linkage. This mechanism is compatible with the possibility contemplated by Spencer

(149) and Suzuki *et al.* (77) that sulfatases may have some sulfotransferase activity. They are not, however, involved in the synthesis of sulfates by the sulfokinase reactions [Baum & Dodgson (150)].

Steroid sulfatases.—The first steroid sulfatase, one hydrolyzing dehydroisoandrosterone sulfate, was found in 1952 in *Helix pomatia* [Henry *et al.* (151)]. Later studies on a corresponding enzyme from *Patella vulgata* by Roy (152, 153) have shown that it is highly specific for 3β-sulfates of 5α or Δ^5 steroids; it is inhibited by sulfate or phosphate ions and has a pH optimum of 4.5.

This enzyme was purified fifty-fold by ammonium sulfate and acetone fractionations. Although it contained much arylsulfatase, the two activities could be separated by paper electrophoresis. Cortisone-21-sulfate was hydrolyzed by the mixture, but inhibition experiments, together with an optimum pH of 5.3 for this substrate, suggested that glyco-, not steroid, sulfatase might be responsible. After electrophoresis, however, the steroid sulfates were all hydrolyzed by material from the same zone, and judgment must be reserved as to how many enzymes are involved and the exact specificity of *Patella* arylsulfatase.

A steroid sulfatase from ox or rat liver has been found to have esentially the same specificity as the above enzyme, but its pH optimum is 7.8 [Gibian & Bratfisch (154), Roy (155)]; this enzyme is in the microsome fraction and seems to be distinguishable from arylsulfatase C on the basis of inhibitors. Estrone sulfate is split, but this may be attributable to the arylsulfatase C.

Mucopolysaccharide sulfatases.—The existence of chondrosulfatase has been known for decades, Neuberg (156). It exists in molluscs [Soda (157)] and in many bacteria [Dodgson & Spencer (117)]; recent studies have been made by Dodgson *et al.* (158, 159, 160) on *Proteus vulgaris*.

In the case of the *Proteus* enzyme, the normally associated sulfatase and chondroitinase have been separated by adsorption of the former on calcium phosphate gel (159). The sulfatase is strongly inhibited by phosphate or fluoride, has an optimum pH of 7, and has no activity toward any native polysaccharide sulfate nor toward substrates of glycosulfatase, arylsulfatase, steroid sulfatase, or myrosulfatase (158). Its action on chondroitin sulfate must be preceded by splitting of the polysaccharide by chondroitinase or by testicular hyaluronidase (159). This forces re-evaluation of previous conclusions that chondrosulfatase is absent from mammalian tissues, since chondroitin sulfate rather than the degraded substrate has been used for testing. This also applies to the question of the enzyme's specificity toward other mucopolysaccharides, as discussed by Dodgson & Spencer (117).

An analysis of the enzymes extractable from acetone powders of *Flavobacterium heparinum* has been made by Korn (161). Only if the organism has previously been adapted to growth on heparin does the extract degrade heparin. There is evidence for the presence of a heat-sensitive sulfamidase, the action of which makes heparin susceptible to breakdown by a glycosidase and a sulfesterase. The latter are present in extracts of both adapted and non-adapted bacteria and can degrade chondroitin sulfate or hyaluronic acid. No fractionation of these enzymes has been reported.

Glycosulfatase.—This enzyme, once known as glucosulfatase because the classical substrate was glucose-6-sulfate, has been found in many species of organisms [Dodgson & Spencer (117)]. It is capable of hydrolyzing mono- and polysulfates of mono- and disaccharides, although its natural substrate is quite uncertain. Molluscs have been studied most, and some properties of the enzyme from *Littorina littorea* are reported by Dodgson & Spencer (162). They find a pH optimum of 5.8 and warn that published data on such enzymes must be under suspicion because of the difficulty of purifying the usual substrate, glucose-6-sulfate. Adenosine-5′-sulfate also is split by glycosulfatase [Egami & Takahashi (163)].

Myrosulfatase.—Mustard oil glycosides, e.g. potassium myronate (sinigrin), exist in mustard seeds together with myrosulfatase and a thioglycosidase. The structure of the substrate has recently been revised by Ettlinger & Lundeen (164). The sulfate is first removed by myrosulfatase; then the glucoside is split to release mustard oil. This sulfatase has been reported (with low activity) in mammalian tissues [Neuberg & Wagner (165)] and is distinct from arylsulfatase, Baum & Dodgson (166).

Alkylsulfatase.—The only example of an enzyme that hydrolyzes a simple alkyl sulfate is recorded by Vlitos (167). He found that the sulfate group of the potential herbicide 2-(2,4-dichlorophenoxy)ethyl sulfate was split off by an enzyme of soil bacteria, in particular by *Bacillus cereus* var. *mycoides*. Nothing is known of the specificity or properties of this enzyme.

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NEUROCHEMISTRY[1]

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In addition to the broad review of this subject by LeBaron (105) last year, the *Symposium on the Biochemistry of the Central Nervous System*, which took place at the Fourth International Congress in Vienna in 1958, has now been published. This, too, deals with a wide range of topics in this field. Another important publication that has appeared in the course of the last year is the monograph by Nachmansohn (131) on the *Chemical and Molecular Basis of Nerve Activity*.

Because of the recent appearance of these various reviews of different aspects of neurochemistry, this chapter will deal only with certain selected topics, which have either developed rapidly in the course of the last year or which have not been so completely covered in other publications. To some extent, this selection is determined by the interests of the authors and also by the necessary limitations of space allocated them. They would also like to apologise for the inevitable omission of important papers, but, in accordance with the expressed wishes of the Editorial Committee, they have not attempted to cover completely the literature even in the selected topics within the field of neurochemistry but have tried rather to deal more completely and critically with certain chosen pieces of published work.

Lipids

Recent reviews (105, 106) have dealt very fully with the many advances in our knowledge of the chemistry of lipid components of nervous tissue, and, in this field, only work subsequent to these reviews will be considered here.

Plasmalogens.—The plasmalogen content of a number of rat, rabbit, and human tissues and of several human neoplastic tissues has been reported by Rapport & Lerner (149), using a new specific method of estimation based on iodination of the unsaturated ether linkage in the aldehydogenic moiety. Absolute values of plasmalogen content of normal organs agree well with those of other workers; the content in the neoplasms approximates the values found for the corresponding normal tissue. It is emphasised that, because of the large variation in total phosphatide content of different organs, the ratio of plasmalogen to lipid phosphorus may be a more useful index than the absolute plasmalogen content in correlating functional changes in an organ with changes in plasmalogen.

The question whether the aldehydogenic group of the plasmalogens in nervous tissue is attached to the α- or β-carbon atom of glycerol has been

[1] The survey of the literature pertaining to this review was concluded in November, 1959.

investigated by Debuch (47) in the ethanolamine phosphatide fraction of ox brain; chimyl and batyl alcohols were identified as the degradation products from reduced plasmalogen, and it was therefore concluded that the aldehydogenic group must be bound to the α position of glycerol in the lipid. However, in view of the conflicting reports of other workers, on using tissues in different species [Gray (70); Marinetti, Erbland & Stotz (123); Rapport & Franzl (148)], further work would seem desirable.

The instability of the ether linkage in plasmalogens to various reagents has been investigated by Bergmann & Liebrecht (14); Fe^{+++}, Fe^{++}, Ag^{+}, Cd^{++}, Au^{+}, BrO_3^{-}, and I^{-} caused breakdown. Thiele (167) found marked spontaneous splitting of the plasmalogens in crude lipid extracts of horse and ox brain by merely incubating an aqueous emulsion of these at 37°, and he has obtained evidence suggesting that this decomposition may be caused by catalysis by a metal complex derived from the original tissue [Thiele (168)]. Gray & Macfarlane (71) had earlier noted a marked breakdown of ethanolamine plasmalogens of ox heart lipids during chromatography on silicic acid columns, which they attributed to the presence of neutral fat. It seems possible that the well-known tendency of plasmalogens to breakdown may be caused by, at least in part, the presence of other materials in the preparations.

Norton (133) has investigated the reaction of $HgCl_2$ with plasmalogens and has shown that this reacts very rapidly with these lipids to form a metallic complex, which can be detected on chromatograms by treatment with diphenylcarbohydrazide.

Sphingolipids.—Sulphatide has been isolated from brain in good yield by a simple and rapid method by Lees *et al.* (108). Analysis of the material showed very close agreement with the theoretical values for potassium cerebron sulphuric acid. Attempts to prepare ceramide and psychosine from this preparation by hydrolysis with an acetic-sulphuric acid mixture and $Ba(OH)_2$, respectively, resulted in considerable decomposition, in contrast to the behaviour of phrenosine [Klenk (93)]. Moreover, the galactoside linkage was found to be labile in the presence of alkali. Sulphur could be released as inorganic SO_4^{--} by treatment with glacial acetic acid at 100°, leaving the cerebroside portion of the molecule intact. The principle of "linked distribution" used in isolating this preparation involves the partition of the lipids in a succession of isolated biphasic systems. At each stage, the sulphatide fraction was concentrated preferentially in one of the phases, and another solvent was added to form the next biphasic system. This process was continued until adequate purification and concentration of the sulphatide had been achieved. The obvious potentialities of this kind of procedure for the fractionation of other lipid mixtures have yet to be explored.

The sulphatide content of human cerebral white matter has been investigated [Austin (11)]; in early childhood and old age, the values found were slightly lower than the normal adult range, while increased levels were found in metachromatic leucoencephalopathy. A method has been developed

for the micro-assay of sphingosine and related bases present in lipids. In this method, the fatty aldehydes, obtained following periodate oxidation of the terminal aminohydroxy portion of the bases, are separated by gas chromatography [Sweeley (166)].

Gangliosides.—Cumings, Goodwin & Curzon (32) have studied the relationship of ganglioside to neuraminic acid and hexosamine in brain. They found a reasonably constant ratio of neuraminic acid to lipid-soluble hexosamine in the cortex in both children and adults and, hence, conclude that either compound is an index of ganglioside content in this tissue. The ratio was less constant in white matter, but they obtained positive evidence for the presence of ganglioside in this tissue also. A method for the direct determination of neuraminic acid in crude brain lipid extracts, which overcomes the difficulty of interference by cerebroside or carbohydrate, has been described by Long & Staples (110).

Fatty acids.—The presence of 2-hydroxystearic acid in a purified sample of phrenosine isolated from bovine spinal cord has been detected by use of reverse phase paper chromatography [Skipski, Arfin & Rapport (162)]. It is suggested that cerebroside preparations in which only C_{24} acids are present are non-representative fractions obtained as a result of solubility differences. Further claims for the presence of lower homologues of cerebronic acid in nervous tissue have been reported [Radin & Kishimoto (147)]; these reports are based on the separation of the mixed cerebroside acids from brain on a florisil column with petrol ether-ether mixtures.

Proteolipids.—A method for the quantitative isolation of the proteolipids from bovine white matter has now been reported by Folch, Webster & Lees (57). The material, which contained little or no free lipid, was obtained by centrifuging an aqueous emulsion of total tissue lipids when crude proteolipid sedimented. This was partitioned in a biphasic $CHCl_3$:MeOH:H_2O system, and the lower phase solutes extracted with ether and then with ethanol. The final residue was readily soluble in wet $CHCl_3$:MeOH and contained 80 to 90 per cent protein, 1.0 to 1.2 per cent P, and 0.5 to 2.0 per cent carbohydrate (as galactose); it accounted for 85 to 100 per cent of the proteolipid protein present in the original tissue extract.

The question of the binding between the protein and lipid moieties of proteolipids has also been studied recently [Webster & Folch (186)]. It has been found that this binding becomes increasingly unstable as the pH of the medium is raised above neutrality in the presence of inorganic salts. This influence of pH and ionic strength on the protein-lipid bonds suggests that the two moieties are joined by salt-type linkages. A somewhat similar instability of protein-lipid bonding in the presence of aqueous salt solutions was observed by Uzman (176) in the isolation of his "neurosclerin" protein fraction from mouse brain.

Histochemical studies by Koenig (100) on the nature of the neurokeratin network of myelin in the spinal cord and peripheral nerves of cats showed that, although displaying some morphological differences, the network has

similar staining and solubility properties in the two tissues; it is thought that, in both tissues, the neurokeratin network consists principally of proteolipid protein. Since only negligible amounts of proteolipid protein have been detected in extracts of peripheral nerve [Finean, Hawthorne & Patterson (54); Folch, Lees & Carr (55)] prepared by methods that extract it from central nervous tissue [Folch, Lees & Sloane Stanley (56)], the probable physicochemical differences between the proteolipids of central and peripheral myelin are underlined.

The possible existence of inositol lipids in nervous tissue yielding inositol monophosphate on hydrolysis was discussed in a previous review by LeBaron (105). The isolation of a phosphatido-peptide from kidney tissue has now been reported [Huggins & Cohn (85)] and the peptide is said to contain monophosphoinositide; it has been claimed on indirect evidence that similar material is also present in brain.

A useful specific procedure for the histochemical demonstration of glycerophosphatides in nervous and other tissues has been described by Adams & Davison (4), utilizing the ferric hydroxamate reaction for ester linkages in aqueous solution. Sphingolipids and triglycerides show no reaction under these conditions because of their hydrophobic properties. A simplified spectrophotometric method for the determination of ester linkages in lipids has also been reported [Snyder & Stephens (164)]. The method is claimed to be linear up to 4 μeq. and to give identical colour yields with a wide range of long-chain fatty acid esters.

The chemical basis underlying the Marchi reaction for degenerating myelin has been studied by Adams (3), using both tissue sections and lipid extracts, and he has suggested that the negative reaction of normal myelin with OsO_4-chlorate is determined by the hydrophilic properties of this tissue, which allow its permeation by chlorate, consequently preventing reduction of the OsO_4 by unsaturated lipids. On the other hand, the hydrophobic properties of degenerating myelin, which may be related to the formation of cholesterol esters, prevent absorption of chlorate. Countercurrent distribution has been applied to the separation of dinitrophenylated and methylated phospholipids [Collins (27, 29)], and the presence in sheep brain of a new complex aminophospholipid has been claimed [Collins (28)].

Lipid Metabolism

Myelinogenesis.—The presence of considerable quantities of esterified cholesterol in developing nervous tissue of the chicken and man has been demonstrated histochemically and chemically by Adams & Davison (5). The percentage of cholesterol present in the ester form was found to reach a peak value at times that varied with anatomical location. The peak in chick embryo spinal cord occurred at the sixteenth day of incubation and in human foetal spinal cord at 34 weeks gestation, when over 40 per cent of the total cholesterol was present as ester. Rather smaller peak values were found in chick whole brain four to six weeks after hatching and in human *corpus callosum* eight weeks after birth. It was concluded that the maximum esterified

cholesterol value coincides with the initial stages of myelination in each anatomical area.

The accumulation of various lipids in the developing nervous system of the rabbit has been studied by Davison & Wajda (45) following their quantitative separation on alumina columns [Long & Staples (111)]. Results confirm those of previous workers cited, except that during myelination there was no transient decrease in brain weight comparable to that reported recently in the mouse by Uzman & Rumley (178).

Korey & Orchen (101) have carried out a detailed study of the deposition of plasmalogen in developing rat brain. The percentage of phosphatide present as plasmalogen at birth was 8.5 per cent and approached almost adult levels of 17 to 20 per cent 60 days postnatally; the most rapid increases occurred between 10 and 22 days after birth.

The nature of the alkali-stable phospholipid in developing rabbit brain has been investigated by Edgar & Smits (51), who were able to confirm the earlier findings of Brante (24) that only a proportion of this fraction consists of sphingomyelin. A similar finding has been reported by Ansell & Spanner (9) in rats.

Marked increases in mucolipid and mucolipid-bound sialic acid in developing chick brain have been described by Garrigan & Chargaff (64). Hydrophobic cerebroside increased more gradually, a finding which is consistent with the earlier suggestion [Uzman & Rumley (178)] that water-soluble glycolipids in myelinating nervous tissue might serve as precursors of cerebroside.

Isotopic studies.—In the great volume of work that has been published during the last two decades on the metabolism of the nervous system lipids, much use has been made of isotopically labelled precursors for studies both *in vivo* and *in vitro*. The wide variety of techniques employed has made it difficult to correlate the results of different authors, so that a great deal of conflicting data has accumulated on this subject. Nevertheless, there has been a general inclination to the view that, contrary to older ideas of their metabolic inertness, many, if not all, of the lipids in nervous tissue are usually in a state of active metabolism.

Recently, several long-term studies, *in vivo*, have been carried out on the metabolism of nervous system lipids and have shown a high degree of persistence of various isotopes incorporated in these lipids during the period of active myelination in the young animal. Davison *et al.* (41) injected 4-^{14}C-labelled cholesterol into the yolk sac of newly hatched chicks and followed its rate of incorporation into liver and brain lipids. The labelled cholesterol was absorbed rapidly from the blood into the liver, reaching a maximum in the first few weeks and then declining to negligible levels at eight weeks. Although absorption into the brain reached a maximum at about the same time, thereafter it showed a remarkably slow decline over the next six months, and a considerable proportion of the labelled cholesterol was still present in the brains of nearly fully grown birds.

Since cholesterol is a constituent of both the myelin sheath and of the

cells of the nervous system, it was suggested that this persistence of labelled cholesterol in adult animals might prove to be more marked in the former site. The work was therefore later extended [Davison *et al.* (42)] to a mammalian species, the rabbit, in which it was possible to separate grey and white matter of the brain. Following the intraperitoneal injection of 4-^{14}C-cholesterol into 17-day-old animals, radioactivity was again found in the brain cholesterol and showed a high degree of persistence for over a year in both grey and white matter, although some decline in activity in the early stages was evident in grey matter. By contrast, the labelled cholesterol had disappeared from heart, liver, kidney, and plasma in the early part of the experimental period. Chemical degradation studies on brain cholesterol [Davison & Wajda (44)] extracted from the tissue one year after injection of the labelled lipid showed that the isotopic carbon was still in the 4 position.

Intraperitoneal injection of ^{14}C-acetate slowly labelled the cholesterol and fatty acids of the brain lipids of young rats [Nicholas & Thomas (132)], whereas little or no labelling was found in adult animals. In this work, too, considerable persistence of labelling was found one year later in both cholesterol and, to a smaller extent, in fatty acids. Similar persistence of radioactivity has been shown for total phospholipids of whole rat brain following injection of ^{32}P into 16 day old animals [Davison & Dobbing (40)] and also in the separated cholesterol and cerebroside and, to a lesser extent, sphingomyelin fractions of rabbit brain after intraperitoneal injection of 3-^{14}C-serine on the eleventh postnatal day [Davison *et al.* (43)]. In this work, grey and white matter were treated separately, and substantially higher activities were found in the latter. The activities of spinal cord and peripheral nerve lipids were comparable to those of brain. Lipid labelling was present in all the separated fractions, showing the diversity of metabolic pathways followed by serine *in vivo*. Labelling of sphingomyelin by ^{32}P, *in vivo*, was found to be more active in young rats than in adult animals [Ansell & Spanner (9)].

This evidence of marked metabolic inertness of at least some of the lipid material in adult nervous tissue, particularly in white matter and in those lipids present in the myelin sheath, strongly suggests that, in metabolic studies on nervous system lipids, consideration must be given to their anatomical site. It would appear possible that certain lipids may be actively metabolized in "cellular" locations, in contrast to the myelin sheath, where normally they may remain comparatively inert once they have been laid down during myelination in the growing animal.

The incorporation of ^{14}C-labelled dimethylamino ethanol, methyl choline, and choline into mouse brain and other organs has been studied *in vivo* for periods of up to 24 hr. by Groth, Bain & Pfeiffer (74). Although choline was a more immediately effective precursor for both acid-soluble and lipid choline, dimethylamino ethanol was ultimately incorporated to a greater extent and retained longer. ^{14}C was excreted from the body more slowly from dimethylamino ethanol than from choline, indicating the slow conversion of the former compound to choline.

D-Glucose-U-^{14}C and D-galactose-1-^{14}C were each incorporated into rat brain ganglioside after intracerebral or intraperitoneal injection [Mandelstam & Burton (122)]. Incorporation was most rapid in six- to ten-day-old animals. Radioactivity was present in ganglioside mainly as galactose or galactosamine and very little as glucose or neuraminic acid.

Moser & Karnovsky (129) found that, in mice killed one hour after intraperitoneal administration of labelled glucose or galactose, the former had been more readily incorporated into brain lipids other than strandin. Strandin was more effectively labelled from galactose. Of particular interest is the finding that glucose as a unit was incorporated into the galactose of brain cerebroside more effectively than was administered galactose. Incorporation of glucose into cerebroside was much more active in young than in old animals; very little change with age was found, however, in the labelling of strandin or total fatty acids. Although the specific activity of cholesterol and cerebroside of adult brain lipids was low, the total activity incorporated was significant.

Virtually nothing is known at present concerning the metabolsim of plasmalogens. Certain lysophosphatides, a type of compound which could be formed by removal of the aldehydogenic group from plasmalogens, are known to have a marked lytic action on certain tissues, including brain [Grosse & Tauböck (73); Webster (185)], and several authors [Debuch (46); Morrison & Zamecnik (128)] have suggested the possible involvement of lysophosphatides in the pathogenesis of demyelinating diseases. The study of plasmalogen metabolism in nervous tissue, particularly from this point of view, would therefore seem to be an urgent problem. Korey & Orchen (101) have shown active incorporation of acetate-1-^{14}C and, to a lesser extent, of palmitate-1-^{14}C into the aldehydogenic moiety following intraperitoneal injection into ten- to thirteen-day-old rats. Palmitate incorporation was markedly suppressed by simultaneous administration of either linoleate or acetate.

A few further observations on plasmalogen metabolism have been reported recently. The ability of a cell-free preparation from rat brain to incorporate 1-^{14}C-palmitate into the aldehydogenic moiety has been described by Gambal & Monty (61); the system required various cofactors, including α-glycerophosphate, cytidine triphosphate, and ethanolamine. In muscle, the incorporation of ^{32}P *in vivo* into the plasmalogens has been studied; the specific activity of the plasmalogen phosphorus was comparable to that of the diacyl phosphatide P, and it was suggested that the formation of the aldehydogenic group occurs on a phosphatide precursor [Schmidt *et al.* (160)]. Growth hormone reduced the plasma plasmalogen levels in adult rats while raising the level in liver [Haven *et al.* (77)]; no change was found in intestine plasmalogen content.

Studies, *in vitro*, of the ability of various ^{14}C-labelled precursors to be incorporated into respiring segments of normal and degenerating cat sciatic nerve have been carried out by Pritchard & Rossiter (144). Acetate, glycerol,

choline, ethanolamine, and serine all entered the total phospholipid fraction. Their order of effectiveness was similar to that found previously for rat brain slices [Pritchard (143)], although the specific activity of nerve phospholipids was less than that of rat brain. ^{14}C-Acetate was also incorporated into cholesterol *in vitro* (Kline *et al.* (95)]. With each of the nitrogen-containing precursors, labelling *in vitro* was confined to the corresponding nitrogenous base of the phospholipids. With ^{32}P, on the other hand, labelling was most marked in phosphatidyl choline, phosphatidic acid, and phosphoinositide, and very little labelling of phosphatidyl serine or phosphatidyl ethanolamine was found [Magee *et al.* (121)]. Nerve section caused an increase in uptake of ^{14}C-labelled precursors into phospholipid (144), although the time course of this increase was different for each precursor. ^{14}C-Acetate incorporation into non-esterified cholesterol was also increased (95), but no labelling of the esterified cholesterol, which appears during degeneration, was observed. An increase in ^{32}P incorporation into phospholipids also occurred after sectioning of the nerve and was mainly caused by increase in specific activity of phosphatidyl choline and phosphatidyl ethanolamine, in contrast to phosphatidyl serine and phosphatidic acid (121).

It is suggested from these studies that, while the stimulation of isotope incorporation produced by sectioning may reflect an increased potential of the nerve to form myelin, it represents also a new pattern of activity of the nerve as a whole, which is possibly associated with alterations in the cells of the myelin sheath.

In nerves regenerating 96 days after crushing (95), this potential for remyelination would appear to be realised, since a large increase in the labelling of phospholipids and non-esterified cholesterol with ^{14}C-acetate was observed; this was apparent when the results were expressed either as specific activities or, in contrast to degenerating nerves, as activity per 100 mg. nerve, indicating a net increase in lipid.

Since activation of acetate to acetyl coenzyme A is required for acetate incorporation into both phospholipids and cholesterol, their effective labelling from this precursor implies that active acetate formation is not deficient in sectioned nerves. Berry & Rossiter (19) have in fact shown that acetic thiokinase is normal at 8 and 16 days, and increased at 32 days following sectioning of nerve; choline acetylase, on the other hand, is markedly decreased. A decrease in choline kinase 32 days after sectioning and the inhibition of the enzyme by phosphoryl choline have also been described [Berry, McPherson & Rossiter (18)].

A slight decrease in phospholipid turnover in frog nerve, as measured by ^{32}P incorporation *in vitro*, apparently results following tetanization [Abood, Goldman & Lipman (2)]. This accompanies a similar decrease in the acid-soluble phosphorus turnover reported earlier [Abood & Goldman (1)].

The biosynthesis of inositol phosphatides by rat brain homogenates has been shown to require Mg^{2+} and cytidine triphosphate in both glycolysing and non-energy-producing systems. Phosphatidic acid augmented the effect

of cytidine triphosphate in the former but was ineffective or inhibitory in the latter [Thompson, Strickland & Rossiter (169)].

Further studies on the enzymic synthesis of phosphatidic acid have been carried out by Hokin & Hokin (82), who found, in brain microsome preparations, two pathways for this synthesis: (*a*) from α-glycerophosphate and coenzyme A thiol esters of fatty acids, a pathway already described in liver [Kornberg & Pricer (103)] and brain mitochondria [McMurray *et al.* (119)]; and (*b*) by the direct phosphorylation of diglyceride by ATP. Deoxycholate in a suitable concentration stimulated synthesis by the second pathway, although it was inhibitory in very low or very high concentrations. The nature of the fatty acids in the diglyceride appeared to be important since the diglyceride from cabbage phosphatidic acid was a much more effective substrate than that from brain lecithin [Hokin & Hokin (79, 82)]. Cabbage phosphatidic acid itself was an effective substrate in the presence of Mg^{2+} to activate the phosphatidic acid phosphatase, which is also present in the microsomal preparations. Earlier work by Hokin & Hokin had shown a stimulating effect by low concentrations of acetylcholine on phosphate exchange by brain phosphatidic acid. Later studies [Hokin & Hokin (80)] indicate that this effect is confined to the pathway utilizing diglyceride and ATP. The acetylcholine effect is blocked by atropine and is dependent on the integrity of the microsome structure, since it is also abolished by freezing, hypotonicity, and deoxycholate. It is suggested that acetylcholine modifies the membrane structure of the microsomes in some way and increases availability of substrate to the diglyceride kinase system. A hypothetical scheme involving breakdown and resynthesis of phosphatidic acid via a diglyceride kinase-phosphatidic acid phosphatase system is proposed as a possible mechanism for the transport of hydrophilic secretory materials across cell membranes [Hokin & Hokin (80, 81)].

The activation and oxidation of fatty acids by rat brain preparations has been found to be rather greater than previously believed [Vignais, Gallagher & Zabin (179)]. Maximum activity was found in the supernatant fraction and considerably less in the particulate fraction, while none could be demonstrated in whole brain homogenates. Stearate and palmitate were the optimum substrates, in contrast to the similar enzyme system in liver, which was described earlier (103), with which laurate gave optimal activity. Activated palmitic acid was oxidized by brain mitochondria at about one-sixth the rate of its oxidation by liver (179). Rat brain supernatant fractions, but not whole brain, can also form palmityl coenzyme A from palmityl adenylate [Vignais & Zabin (180)]; a palmityl adenylate hydrolase is present in acetone-dried preparations of rat brain.

The incorporation of mevalonic acid into rat brain cholesterol *in vitro* has been shown by Korey & Stein (102). Immature brain tissue was much more active than adult, while white matter from the brain stem of sixteen-day-old rats was five times as active as grey matter. Brain slices incorporate mevalonic acid more slowly than acetate into cholesterol and fatty acids, the re-

verse being found with liver [Garattini, Paoletti & Paoletti (62)]. 2^{14}C-mevalonic acid was poorly incorporated into brain cholesterol and fatty acids *in vivo* [Garattini, Paoletti & Paoletti (63)].

Proteins

Chemistry.—Our present knowledge of the chemistry and metabolism of the proteins of the nervous system has been extensively reviewed in the previous orticle in this series by LeBaron (105) and also by Richter (151) and Waelsch (181). Since that time, reports of further work in this field of neurochemistry have appeared and will be reviewed here.

The conditions used by different workers for the extraction of soluble brain protein vary considerably, and LeBaron & Folch (107) have studied the conditions necessary for maximal extraction of this fraction. They found that maximal extraction occurs at ionic strengths between 2.5 and 2.0 at pH 6 to 9. In these circumstances, fractions were obtained amounting to 47 per cent and 21 per cent of the total protein of grey and white matter, respectively. Dialysis of these extracts at 4°C. against distilled water caused precipitation of globulin fractions containing 25 per cent lipid, of which 25 per cent was cholesterol. Lipid-free albumin was left in solution. The compositions of these protein fractions were similar in both grey and white matter, although the yield from the latter tissue was the smaller, and the authors suggest that these observations would seem to indicate that the bulk of the material in the soluble protein fraction from white matter consists of intracellular, rather than myelin, constituents and, thus, is derived from glial rather than neural elements. The proteins of rat brain have been isolated in seven fractions by Dingman, Sporn & Davies (49). Some differences in lipid and amino acid composition and marked differences in the uptake of ^{14}C-labelled proline *in vivo* were were found between the fractions.

The electrophoretic properties of brain proteins following solubilization of the tissues with lysolecithin solution have been studied by Gent (66). The ability of lysolecithin to clear aqueous brain homogenates completely has been studied by Webster (185) and will be discussed in more detail below. The use of such cleared homogenates for the study of brain proteins is of considerable interest, since it offers the possibility of investigating electrophoretically those proteins which are normally insoluble in aqueous buffers and which therefore require relatively more drastic procedures for their isolation. Gent (66) obtained four peaks from aqueous brain extracts without the use of lysolecithin, together accounting for about 30 per cent of the total brain protein; extracts obtained with the aid of lysolecithin in increasing amounts caused a progressive increase only in the so-called albumin peak, accompanied by separation of the ascending limb into several components of not very different mobilities. It was concluded that the peak showing the increase following treatment with lysolecithin is probably caused by structural proteins.

Present knowledge of changes in brain protein in disease is scanty. Karcher, van Sande & Lowenthal (89) have studied the electrophoretic pat-

terns of soluble brain and cerebrospinal fluid proteins by a micro-technique on agar gel. The procedure appears to give better resolution than electrophoresis on paper, and differences in protein composition were found in different parts of the brain. An abnormal distribution of proteins, including a relative increase in γ-globulin, was observed in both cerebrospinal fluid and brain tissue from cases of subacute sclerosing leuco-encephalitis. Further studies on the electrophoretic properties of soluble brain proteins have been carried out by Kiyota (91), who has shown that high albumin and α-globulin levels occur particularly in immature brain and have an important part in determining the water content of brain tissue. A similar increase in albumin and α-globulin was found also to occur in patients with a tendency to convulsions, including idiopathic epilepsy [Kiyota (90)], and it is suggested that brains from individuals with this condition may contain immature structural components, which confer an increased liability to oedema and, hence, to convulsions. Similar studies of the soluble proteins of oedematous brains [Kiyota (92)] indicated two types of oedema: a simple type in which the water content alone was increased and a more complex type in which albumin and α-globulins were also raised.

Metabolism.—Studies on the phosphoproteins of brain have been continued by Heald. In his previous work in this field, Heald showed that proteins which incorporate ^{32}P more actively following electrical stimulation of brain slices, *in vitro*, are probably intermediates in metabolic reactions involving phosphate transfer. Two major phosphoprotein fractions have now been described in guinea pig brains [Heald (78)], one soluble and the other insoluble in isotonic sucrose. *In vivo*, the soluble fraction was the more active as regards ^{32}P incorporation. In experiments with slices, *in vitro*, on the other hand, the soluble phosphoprotein was partly lost by solution in the medium, and the insoluble phosphoprotein showed the higher rate of incorporation. Stimulation of brain slices, *in vitro*, with electrical pulses caused an increase mainly in the specific activity of the insoluble phosphoprotein, and evidence has been put forward showing that this is concentrated in particles of the nuclear fraction. The stimulation of respiration and glycolysis of brain slices by electrical pulses has been shown to be inhibited by certain histone preparations, and it has tentatively been suggested that this effect may be caused by combination of the histone with those phosphoproteins implicated in phosphate transfer mechanisms [McIlwain (116)].

Recent work on the incorporation of ^{35}S-methionine into proteins of various isolated cell fractions from rat brain has been reported by Clouet & Richter (26). In agreement with other workers [Furst, Lajtha & Waelsch (60)] using labelled lysine, the highest rate of uptake of methionine was found in the microsomal fraction. The most rapid incorporation occurred in a lipoprotein corresponding to the Nissl granules; the half life of the protein is estimated at 50 to 100 min.

Evidence suggesting that adult rat brain can itself convert proline to glutamic acid, aspartic acid, alanine, arginine, ornithine, and γ-aminobutyric acid has peen presented by Sporn, Dingman & Defalco (165) and would seem

to imply a greater capacity for amino acid metabolism in brain than earlier findings suggested [Waelsch (182)]. Roberts, Flexner & Flexner (153), on the other hand, found that adult brain, unlike that of newborn animals, is unable to incorporate glucose carbon into essential amino acids. These workers also found, in both newborn and adult animals, a lack of parallelism between the ratios of the rates of incorporation of several amino acids and the ratio of their concentrations in tissue proteins. On this evidence, they tentatively suggested that amino acids may be incorporated into brain proteins partly by exchange reactions and not only during synthesis of complete molecules.

The observation by Sporn, Dingman & Defalco (165) that brain can convert proline into ornithine and arginine led them to speculate further whether brain is also able to hydrolyse arginine to ornithine and urea and, if so, whether urea formation by this route might be a means by which brain can detoxify ammonia. In this connection, it is of interest that a hitherto unknown form of mental deficiency has recently been described [Allan *et al.* (8); Dent (48)] and is associated with the excretion of large amounts of argininosuccinic acid. This substance is known to be an intermediate in the ornithine cycle in certain non-nervous tissues [Ratner (150)], but since, in these mentally deficient patients, the blood urea levels were normal and the levels of argininosuccinic acid in the cerebrospinal fluid were greater than in the plasma, it has been suggested that the substance may be formed in these cases as a result of a metabolic block in some as yet unknown synthetic process in the brain. Further work on the so-called "maple syrup urine disease," involving mental deficiency and a presumed metabolic block in the metabolism of leucine, isoleucine, and valine, has been published by Dancis *et al.* (37), Dancis (36), and Mackenzie & Woolf (118).

The effect of a number of oxidizable substrates on ammonia formation in brain has been studied [Guha & Ghosh (75)], and the possible mechanisms of the action of glucose in inhibiting ammonia formation has been discussed. Tower (175) has reviewed the subject of glutamic acid metabolism in the mammalian central nervous system, while Elliott (52) and Roberts & Baxter (152) have published further reviews on γ-aminobutyric acid in brain. Irreverre & Evans (86) have reported the isolation of γ-guanidinobutyric acid in crystalline form from calf brain.

Copper Metabolism

Interest in the field of copper metabolism in the central nervous system has been maintained, and a number of papers have appeared on the more fundamental aspects of these problems, as well as on the disturbance of copper metabolism that occurs in hepatolenticular degeneration.

Porter & Ainsworth (139) have continued the study of the copper proteins isolated earlier in brain by Porter & Folch (140, 141). It had been shown that cerebrocuprein I reacts directly with sodium diethyldithiocarbamate, whereas both caeruloplasmin and hepatocuprein fail to react directly. It has now been shown that less than 15 per cent of the copper in crude brain cere-

brocuprein I and in Fraction I copper proteins from normal human brain reacts directly with sodium diethyldithiocarbamate. In brains from two cases of hepatolenticular degeneration, on the other hand, 29 to 45 per cent of the Fraction I copper reacted directly. This excess of direct-reacting copper may represent an abnormal accumulation of copper bound to brain proteins that are normally copper-free.

A brief description of an improved method for the preparation of caeruloplasmin from Fraction IV prepared by the Cohn cold ethanol procedure has been given by Sanders, Miller & Richard (156). The blue α-globulin is extracted from Fraction IV with 0.06 to 0.1 *M* NaCl followed by adsorption onto diethylaminoethylcellulose, from which it is eluted with more concentrated sodium chloride. Curzon & Vallet (35) have also described a preparation of caeruloplasmin from a globulin fraction of human plasma.

An interesting report indicating that the copper can be reversibly dissociated from caeruloplasmin has appeared [Morell & Scheinberg (127)]. Although the physiological significance of this blue copper protein is obscure, it has sometimes been assumed that it is connected more with its ability to act as an oxidase than with any transport function involving reversible binding and release of the copper from the apoprotein, since attempts to obtain dissociation of the copper from the protein have, in the past, resulted in irreversible denaturation. The preparation of what would appear to be the native apoprotein, together with the regeneration of a copper-containing protein having 60 per cent of the blue colour and tightly bound copper and 86 per cent of the enzymic activity of the original caeruloplasmin, would suggest that this protein may have some function in reversibly binding and releasing copper in the organism.

Curzon (33) has studied the effect of digestion with chymotrypsin on the oxidase activity and non-dialysable copper content of caeruloplasm in preparations. His findings suggest that only four of the eight copper atoms in the caeruloplasmin molecule are in the region that undergoes proteolysis by chymotrypsin. In this connection, it is of interest to recall that Scheinberg & Morell (159) had earlier shown that only half the copper in caeruloplasmin is exchangeable with ^{64}Cu.

A study of the factors that influence serum copper levels and caeruloplasmin oxidative activity in the rat has been made by Meyer, Meyer & Horwitt (126). Using *p*-phenylenediamine as substrate, they have shown that in both control and adrenalectomised rats, injections of turpentine, oestradiol benzoate, and adrenaline cause an increase in the serum copper level and in oxidase activity. They have suggested that the slight elevation of the serum copper level claimed to be present in many patients suffering from mental disorders may be a reflection of an increase in the level of circulating adrenaline.

Aprison & Hanson (10) have studied the mechanism of inactivation of oxidase activity of serum and of purified caeruloplasmin by ultraviolet irradiation at 2537 A. They have concluded that loss of enzymic activity is

correlated with a change in the state of "bound" copper caeruloplasmin to "unbound" copper.

A comparative study of different histochemical methods for the detection of copper in cells has been carried out by Howell (84). The methods that he has studied are based on the use of (*a*) sodium diethyldithiocarbamate, (*b*) rubeanic acid, (*c*) *p*-dimethylaminobenzylidinerhodanine, and (*d*) *sym*-diphenylcarbazide. He found that with livers all four tests gave positive results when the copper content of the tissue exceeded 100 μg./gm., wet weight. With levels between 80 and 100 μg./gm., variable results were obtained, while, when the copper content was below 80 μg./gm., all four methods gave negative results, with an occasional exception in the case of the sodium diethyldithiocarbamate procedure. He has concluded that this latter procedure is the most sensitive and reliable of the four methods. In hepatolenticular degeneration, copper granules can be seen in the liver cells. He examined many different areas of the brain from patients with this condition; in some instances, a very faint diffuse staining was seen in sections from the basal ganglia, but in no case were definite granules seen as in the liver. Each of the four methods gave strongly positive reactions for the copper in the Kayser-Fleischer ring that occurs in the eyes of patients with this disease.

Howell & Davison (83) have recently extended the earlier work on tissue copper levels in swayback lambs. Although Shearer, Innes & McDougall (161) had earlier claimed that the copper level in the brain of swayback lambs was normal, Howell & Davison found a significant reduction in both brain and liver levels; kidney tissue, on the other hand, showed no reduction in copper content, a finding which they have tentatively attributed to the fact that before slaughter the animals had been fed away from the home farm for some time on food with a, presumably, normal copper content. They also found that the cytochrome oxidase activity was reduced in the brains of affected animals.

Papers have appeared on the genetic aspects of Wilson's disease [Bearn (12)], on its treatment with 2,3-dimercaptopropanol, penicillamine, and Versene [Cumings (31)], and on the chemistry and biochemistry of caeruloplasmin [Curzon (34)], the latter including a brief review of recent work on some of the substances that caeruloplasmin is capable of oxidizing.

Walshe (183) has provided a useful and critical review of current theories on the pathogenesis and treatment of Wilson's disease. Sass-Kortsak *et al.* (157) have made a careful study of two brothers suffering from Wilson's disease, with Kayser-Fleischer rings and increased urinary excretion of copper, but with normal blood levels of caeruloplasmin. Despite this, one of the two patients did show a marked delay in the rate of incorporation of ^{64}Cu into the caeruloplasmin. The authors have provided an interesting discussion of these findings in the light of the currently held views concerning the pathogensis of this disease. Reports dealing more with the diagnostic and therapeutic aspects of Wilson's disease have appeared by Blattner (22), Francois,

et al. (58), Gastager & Tschabitscher (65), Knovalov & Gotovtseva (98), Lygren (113), Lygren, Sörensen & Bernhardsen (114) and Warnock & Neill (184).

Biochemistry of the Cerebrospinal Fluid

Reference must be made to some of the recent work on the biochemistry of the cerebrospinal fluid since, apart from the review by Green (72), this topic does not appear to have been covered in recent publications.

Interest in the biochemistry of the cerebrospinal fluid is, of course, of continuing and increasing importance to clinical biochemists, particularly in view of the improved micro-methods for the determination of many constituents of this fluid and the wider range of constituents that can now be examined. As an example of the breadth of analytical work that is now being carried out on samples of cerebrospinal fluid from patients with various neurological and psychiatric disorders, a recent publication by Plum & Fog (138) summarises their findings with fluid obtained from patients with multiple sclerosis as regards, potassium, calcium, magnesium, phosphate, chloride, iron, various protein fractions, lipoproteins, phospholipids, cerebrosides, cholesterol, glutamic-oxalacetic transaminase, true and pseudocholinesterase, and neuraminic acid.

A number of papers have appeared recently describing improved methods for the determination of various constituents of cerebrospinal fluid.

Roboz *et al.* (155) have described methods for the determination of total lipids, free and esterified cholesterol, and total phospholipids in chloroform-methanol extracts of 10 ml. samples of cerebrospinal fluid.

Tourtellotte *et al.* (174), impressed with the need for quantitative microchemical techniques requiring only small volumes of cerebrospinal fluid, have described a micro-method capable of determining 2 μg. of lipids with a coefficient of variation of about 7 per cent. Because of the very small amounts of phospholipids present in normal CSF, Tourtellotte, Parker & DeJong (172) have worked out a micro-method, based on the work of Lowry and his associates (112), for the determination of 0.02 μg. of phospholipid with a coefficient of variation of about 5 per cent. A micro-method capable of determining 0.5 μg. of total cholesterol and 1 μg. of free cholesterol in cerebrospinal fluid (thus requiring less than 2 ml. of normal cerebrospinal fluid) has been reported by Tourtellotte, Skrentny & DeJong (173). Using these methods, Tourtellotte (171) has described the "normal lipid profile" of cerebrospinal fluid.

Papadopoulos, Cevallos & Hess (135) have identified both the free and esterified fractions of cholesterol in cerebrospinal fluid by paper chromatography and have described a micro-procedure for their extraction, chromatographic separation, and colorimetric determination by the $FeCl_3$-H_2SO_4 reagent. Using this technique, Green *et al.* (72a) have reported on the levels of

free and esterified cholesterol in the cerebrospinal fluid in a variety of neurological diseases. Unfortunately, there are wide discrepancies in the levels of free and esterified cholesterol in the cerebrospinal fluid as reported by different workers. These discrepancies are almost certainly attributable to the different methods of estimation and, in view of the importance attached to the estimation of cerebrospinal fluid cholesterol levels as a possible index of the activity of multiple sclerosis and other demyelinating conditions [Poser & Curran (142)], it is of value that these micro-methods are now being examined.

Because of the increase in the γ-globulin content of the cerebrospinal fluid that occurs in various neurological diseases, Karcher, van Sande & Lowenthal (89) investigated methods for a more complete separation of the γ-globulins than can be obtained by paper electrophoresis. They have found that electrophoresis in an agar gel, although causing no change in the γ-globulin in normal cerebrospinal fluid, shows a striking improvement in the separation of the abnormal γ-globulins in pathological fluids; in some conditions, for example in subacute sclerosing leuco-encephalitis, the γ-globulins appeared as four different fractions with varied, although overlapping mobilities.

Knauff (96) has examined the content of free amino acids in cerebrospinal fluid by use of high-voltage paper electrophoresis followed by paper chromatography and has detected the presence of 25 amino acids and related ninhydrin-reacting substances. He has also examined a number of pathological fluids but has not found any changes in the pattern of the free amino acids that would appear to be of diagnostic significance. In an additional paper, Knauff & Zickgraf (97) have identified the presence of free ethanolamine in human cerebrospinal fluid; it occurred regularly in all the 30 samples studied and in a concentration varying from 0.5 to 1.5 μg./ml.

The chromatographic identification of glutamine in cerebrospinal fluid and its estimation by the method of Whitehead & Whittaker (187) have been described by Gilon *et al.* (69); they have confirmed the earlier finding of high glutamine levels in hepatic cirrhosis and hepatic coma.

Further papers have appeared on the neuraminic acid content of the cerebrospinal fluid. Bogoch (23) has described the preparation of neuraminic acid from brain gangliosides and the detailed procedure for the estimation of neuraminic acid in the cerebrospinal fluid by a modification of the method of Klenk & Langerbeins (94). He has shown that the mean neuraminic acid content of the cerebrospinal fluid in schizophrenic patients is considerably below that of non-schizophrenic adults. It must be pointed out, however, that there is a very considerable difference between the absolute values reported by him and those described by Roboz *et al.* (154). Plum & Fog (138) have also reported briefly on the neuraminic acid levels in the cerebrospinal fluid in multiple sclerosis; a slight increase was found in this condition, although it was concluded that the increase is not statistically significant. The levels found by Plum & Fog agree well with those of Roboz *et al.* (154).

Uzman, Bering & Morris (177) have now extended their observations on the neuraminic acid concentration in the cerebrospinal fluid to 262 cases. They have shown that the neuraminic acid is present in two forms, a protein-bound fraction, the concentration of which increases with the protein concentration, and a dialyzable fraction with a concentration that is highest in the ventricular fluid.

Chapman & Wolff (25) have described some interesting experiments from which they have concluded that the cerebrospinal fluid from patients with progressive disease of the nervous system contains detectable amounts of a proteinase capable of acting on plasma proteins (bovine globulin, Fraction II) to form polypeptides of the bradykinin type. The possible implications of this work in connection with disease of the central nervous system are of interest, particularly in view of the authors' conclusion that these peptides have vasodilator properties.

Bernsohn, Boshes & Possley (15) have carried out a detailed study of the levels of true and pseudo-cholinesterase and of aliesterase in cerebrospinal fluid obtained by lumbar puncture from 159 patients with various neurological diseases. In the course of this work, they have made the perhaps somewhat surprising finding that the level of the true cholinesterase is increased in patients with myasthenia gravis. It is true that they studied only seven myasthenic subjects, but, in view of the classical thinking that myasthenia gravis is essentially a disorder at the level of the motor end-plates in the muscles, it seems important that this work should be extended.

The earlier work on the levels of glutamic-oxalacetic transaminase in the cerebrospinal fluid has been admirably reviewed by Green (72), and, although further confirmatory reports have been published, little that is new has appeared since. Plum & Fog (138) have failed to detect any significant abnormality of the transaminase levels in the cerebrospinal fluid in multiple sclerosis.

Albrechtsen, Storm & Claassen (6) have studied the fibrinolytic system in human cerebrospinal fluid. They have been able to show that cerebrospinal fluid contains a precursor of a plasminogen activator but does not normally contain plasminogen or a trypsin inhibitor.

Esterases and Cholinesterases

The localization of the cholinesterases in the nervous system has continued to attract the attention of a number of groups of workers, and, in particular, information is now accumulating concerning their intracellular localization.

Aldridge & Johnson (7), for example, have carried out a carefully controlled study of the distribution of both true and pseudo-cholinesterase in rat brain. Homogenates of brain have been separated into five subcellular fractions by differential centrifugation; the degree of contamination of different fractions by mitochondria and nuclei has been assessed by simultaneous determinations of succinic dehydrogenase and deoxyribonucleic acid re-

spectively. They have found that 35 to 40 per cent of the true cholinesterase is sedimented in the microsomal fraction, 27 per cent appears in the heavy mitochondrial fraction, 14 per cent with the light mitochondria, 9 per cent in the supernatant fraction, and 6 per cent in the nuclear fraction.

Pseudo-cholinesterase activity, on the other hand, is highest in the nuclear fraction (36 per cent), while 27 per cent is found in the supernatant fraction. These workers also examined the distribution of an esterase hydrolysing phenyl butyrate and found it to be similar, but not identical, to that of cholinesterase; in addition, they have shown that 35 per cent of the glutathione reductase activity is present in the mitochondrial and 44 per cent in the supernatant fractions.

Toschi (170) and Hanzon & Toschi (76) have also published interesting and detailed accounts of their study of the distribution of cholinesterase in brain fractions by use of both biochemical techniques and electron microscopy. They, too, have found that the highest concentrations of cholinesterase occur in the microsome fractions. They have shown further that this enzyme is mainly associated with the membranous structures rather than with the particles that make up most of the microsome fraction. A similar association of cholinesterase with membranous structures in subcellular fractions derived from the electric tissue of *Torpedo* has also been reported by Frontali & Toschi (59).

Using a modification of Koelle's thiocholine histochemical method and also the Cartesian diver technique, Giacobini & Holmstedt (68) have studied the cholinesterase content of neurones in the antero-lateral group of the anterior horn of the spinal cord of the rat. The large motor neurones stain heavily, and some staining is also visible along the neurites emerging from them. Single anterior horn cells were isolated from the antero-lateral group by micromanipulation techniques, and their cholinesterase activity was measured in a micro-diver. By this means, the authors have been able to describe two different groups of cells, one having a true cholinesterase activity per cell four times higher than the other.

In view of the recognised limitations of differential centrifugation techniques, particularly when applied to tissues such as the central nervous system where there is apt to be gross inhomogeneity of the preparation with respect to cell type, Giacobini (67) has also applied the Cartesian diver technique to a study of the determination of cholinesterase levels in samples of intracellular material derived from isolated anterior horn cells from the cervical region of the rat spinal cord. The technical details of his procedure are described fully, and he has already been able to show that a sample of cytoplasm hydrolysed acetylcholine at about 10 times the rate shown by a sample of nucleoplasm.

A histochemical study of the effect of denervation on both true and pseudo-cholinesterases in the rat adrenal gland has been published by Eränkö, Hopsu & Räisänen (53). After division of the splanchnic nerves, true

cholinesterase activity disappears from the nerve trunks and fine medullary nerve net of the adrenal medulla. Pseudo-cholinesterase activity and its distribution are practially unaffected by splanchnic denervation.

Snell (163) has followed up his earlier histochemical work on cholinesterase activity in degenerating peripheral nerve with a study of the distribution of cholinesterases in the superior cervical ganglion of the cat, and of the effect on this of sectioning of the preganglionic nerve trunks.

Further papers on the localization of cholinesterase in autonomic ganglia and on the effects of inhibition of cholinesterase on ganglionic transmission have appeared by Koelle & Koelle (99) and McIsaac & Koelle (117). Loewenstein & Molins (109) have demonstrated the presence of cholinesterase in Pacinian corpuscles dissected from the mesentery and pancreas of the cat. Quite high activities are present, a single corpuscle hydrolysing about 1.6 μg. acetylcholine per hour; butyrylcholine is hydrolysed at about three times the rate of acetylcholine.

A comparative study of the esterase activity in human brain and serum has been carried out by Bernsohn, Possley & Liebert (16). They have shown that at least four different eserine-resistant esterases occur in these tissues, namely (*a*) a Ca^{2+}-dependent enzyme in serum which hydrolyses phenylacetate, (*b*) an enzyme in brain which hydrolyses phenylbutyrate, and (*c*) and (*d*) at least two different enzymes in serum and in brain which hydrolyse phenylacetate and phenylbutyrate. They have further shown that high phenylacetate-hydrolysing activity appears to be associated more with the grey matter.

Lawler (104) has described a simplified procedure for the partial purification of the true cholinesterase from electric tissue; more than 200-fold purification has been achieved with a 20 per cent yield. A further report has appeared on a colorimetric micro-method for the determination of cholinesterase activity by use of acetylthiocholine as substrate [McOsker & Daniel (120)].

The chemical nature of the di-isopropyl fluorophosphate binding site of pseudo-cholinesterase has been studied by Jansz, Brons & Warringa (87); they have used a pseudo-cholinesterase preparation from horse serum purified 1000 times the original serum. After complete inhibition with di-isopropyl fluorophosphate-^{32}P, followed by dialysis to remove unreacted inhibitor, the preparation was digested with pepsin, and the peptide mixture separated out chromatographically. A ^{32}P-containing peptide was recovered and was shown to be homogeneous both by paper chromatography in several solvents and by paper electrophoresis. Analysis of this peptide indicated that it had the following structure:

$$\begin{array}{c} \text{Phe-Gly-Glu-Ser-Ala-Gly (Ala}_2\text{-Ser)} \\ | \\ \text{P} \end{array}$$

The sequence Gly-Glu-Ser-Ala-Gly is the same as that found in the di-isopropyl fluorophosphate binding site of horse liver ali-esterase [Jansz, Posthumus & Cohen (88)] and has some similarity to the binding sites that have been described in thrombin, chymotrypsin, and trypsin, all of which show the same sequence dibasic amino acid-Ser.

It has been known for some time that cholinesterase, following inhibition by certain organophosphorus compounds, is converted on storage from a form reactivatable by oximes into a non-reactivatable form. This "ageing" process of di-isopropylfluorophosphate-inhibited cholinesterase would appear to be associated with a conversion of the "di-isopropyl-phosphorylated" enzyme into a "monoisopropyl-phosphorylated" form by loss of isopropanol [Berends *et al.* (13)].

Information concerning the parts played by spontaneous hydrolysis, ageing, and the synthesis of new enzymes in the recovery of sheep erythrocyte and rat brain cholinesterases following inhibition *in vivo* with various organophosphorus anti-cholinesterases has been given by Blaber & Creasey (21).

Work on the antidotal actions of oximes and hydroxamic acids against organophosphorus anti-cholinesterases has advanced. A useful review by Davies & Green (38) on the chemotherapy of poisoning by these compounds has appeared: the detailed nature of the biochemical lesions in poisoning by compounds of this type is discussed, together with the rationale on which the development of oxime therapy rests together with the results of treatment both in animals and in man.

Further papers on the role of "molecular complementariness" in the designing of compounds to reactivate alkylphosphate-inhibited enzymes have appeared by Wilson (188) and Wilson, Ginsburg & Quan (189), the former in particular giving a clear and interesting account of the theoretical considerations.

The mechanism of the antidotal action of diacetylmonoxime has been studied in detail by Myers (130) with interesting results. It had been known that diacetylmonoxime shows various anomalous features as an antidote against sarin.[2] Myers has now shown that administration of diacetylmonoxime to sarin-poisoned rats causes only partial reactivation of the cholinesterases but an almost complete reactivation of the plasma ali-esterase activity. He has further shown, by pretreatment of rats with tri-*ortho*-cresyl phosphate (to cause 95 per cent inhibition of the plasma ali-esterase activity), that the greater part of the amount of sarin required to kill a rat is evidently used up in causing inhibition of the plasma ali-esterase. His results support the conclusion that the effectiveness of diacetylmonoxime is largely attributable to the reactivation of a non-essential ali-esterase, so that, in effect, it catalyses the destruction of sarin in the blood before it reaches the vital

[2] Sarin: isopropylmethylphosphorofluoridate.

centres of the animal. Myers suggests that a possible mechanism would be a cyclic process involving repeated inhibition and reactivation of the non-essential plasma esterase.

Other papers on oxime therapy have appeared from Berry, Davies & Green (17), Crook, Koon & Oberst (30), Davies, Green & Willey (39), Edery & Schatzberg-Porath (50), O'Leary, *et al.* (134), and Scaife (158).

The distribution of phospholipase B in mammalian tissues has been studied by Marples & Thompson (124). They have shown that activity of this enzyme is readily demonstrable in simple aqueous homogenates of a number of animal tissues. Nervous tissue contains only a low level of activity, but the grey matter of the central nervous system is considerably more active than the white matter.

Although phospholipase A activity has not as yet been demonstrated with assurance in nervous tissue, a considerable amount of work has been carried out recently on the action of phospholipase A (and of the lysolecithin that can be formed by its action) on brain tissue. In part, this work has been stimulated by the fact that certain snake venoms, known to contain phospholipase A, exhibit neurotoxic properties and also by the demonstration that both phospholipase A and lysolecithin can cause demyelinating changes in central nervous system tissue [Birkmayer & Neumayer (20); Morrison & Zamecnik (128)]. Much of the recent work has been carried out by Quastel and his colleagues [see Quastel (145); Quastel & Scholefield (146)]. Petrushka, Quastel & Scholefield (136) have shown that, when snake venom (heated to inactivate enzymes other than phospholipase A) is added to suspensions of brain, liver, or kidney mitochondria, an initial stimulation of the rate of respiration occurs, followed by a rapid fall; they have also made the interesting observation that addition of the heated venom to rat brain cortex slices causes a delayed fall in oxygen uptake, whereas this does not occur with kidney or liver slices. In a further paper, they have studied the uncoupling of oxidative phosphorylation that can be induced in mitochondrial preparations by heated snake venom and have shown that various phospholipids can exert a specific protective action against this effect [Petrushka, Quastel & Scholefield (137)].

Marples, Thompson & Webster (125) have studied the action of lysolecithin on brain homogenates. They have shown that homogenates "cleared" by this compound contain a number of enzymes (true and pseudo-cholinesterase, alkaline phosphatase, glycerylphosphorylcholine diesterase, proteinase, and glutamic-oxalacetic transaminase) in a fully active state, although other enzymes, as was known from the earlier work of Quastel and his colleagues, are partially inactivated.

McArdle, Thompson & Webster (115) have also carried out a study of the action of snake venom phospholipase A (and of lysolecithin) on brain slices respiring in a glucose-containing medium. They have found that the action of each of these substances causes a rapid and extensive release of the intra-

cellular glutamic-oxalacetic transaminase into the surrounding fluid. Both true and pseudo-cholinesterases are also released from the slices by these agents, although to a smaller extent than the glutamic-oxalacetic transaminase. In confirmation and extension of the results of Petrushka, Quastel & Scholefield (136), they have found that the respiration of rat brain slices is more sensitive to inhibition by snake venom than is the respiration of either liver or diaphragm.

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NUTRITION[1,2]

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In accordance with the wishes of the editors, this review is restricted to a few topics, which appear to be particularly timely and in a state of rapid development.

Selenium and Vitamin E

The role of selenium in animal nutrition assumed an entirely new aspect when Schwarz & Foltz (1) announced that concentrates of "Factor 3," which prevented dietary necrotic liver degeneration in rats, contained selenium and that feeding of small amounts of sodium selenite, like vitamin E, also prevented liver necrosis. This was emphasized when Patterson *et al.* (2) reported, shortly thereafter, that selenite also prevented exudative diathesis in chickens. Whereas, heretofore, selenium was considered only from the standpoint of its toxicity (3, 4, 5), it has now become apparent that traces of selenium compounds may have important, perhaps unique, nutritional and metabolic functions. It has been suggested, in fact, "that selenium is an essential trace element" (1, 6), although the evidence on this point is not decisive at present.

Syndromes of malnutrition affected by selenium.—Schwarz (7) summarized the sequence of changes that occurs in dietary necrotic liver degeneration as revealed by macroscopic, microscopic, electron microscopic, and some biochemical observations. In the Fischer strain of rats, during the third week of feeding the deficient diet, liver slices lost their capacity to maintain for more than about 30 min. a high initial rate of oxygen consumption *in vitro* (8); i.e. they developed the so-called respiratory decline. The terminal phase, when macroscopic necrotic changes in the liver became apparent, lasted only one to two days and was accompanied by hypoglycemia, very low glycogen content of the liver, increased blood lactate, and convulsions (9). Intravenous infusion of glucose at this time abolished convulsions but did not prevent a fatal gradual fading of respiration and of the heartbeat.

The respiratory decline in liver has also been observed by Hock & Strunz (10). It was particularly pronounced when succinate was used as a substrate (11). According to Schwarz *et al.* (8), the development of the respiratory decline requires a concomitant deficiency of both selenium and vitamin E; this is in line with his suggestion (7) that two different metabolic systems, acting

[1] The survey of the literature pertaining to this review was concluded in August, 1959.

[2] Paper No. 1030 Miscellaneous Journal Series, Minnesota Agricultural Experiment Station.

through alternate pathways, must be deranged to establish the initial biochemical and subsequent morphologic lesion. Other metabolic abnormalities are an early onset of increased basal metabolic rate, increased fixation of ^{131}I in the thyroids, and a decreased excretion of ^{131}I (12). Olson & Dinning (13) observed decreased coenzyme A activity in livers of rats with acute dietary necrosis. The addition of 5 μg. of selenium as sodium selenite to 100 gm. of a necrogenic diet greatly increased the incorporation of ^{35}S from labeled cysteine into coenzyme A of the liver without concurrent increase in the labeling of liver proteins (14). The same dietary treatment also largely prevented a decrease in mitochondrial reduced diphosphopyridine nucleotide cytochrome-*c* reductase, in pyruvate oxidase, and in the P:O ratios noted in liver preparations of rats fed the necrogenic diet (14).

Mascitelli-Coriandoli *et al.* (15) produced carbon tetrachloride poisoning in rats fed a normal, presumably adequate diet supplemented with 0.6 mg. Se (Na_2SeO_3) per kg. The creatine content of the liver and its capacity to synthesize creatine from guanidoacetic acid or from methionine and guanidoacetic acid were much less depressed than when no selenium was added to the diet. The effect of this treatment on the fat content of the liver was not reported.

When a vitamin E-deficient diet containing 30 per cent torula yeast was fed to mice (16), a syndrome of multiple necrosis developed and was fatal in 65 to 73 days; it could be prevented by the feeding of α-tocopheryl acetate, a "Factor 3" concentrate, or sodium selenite.

Swine that had been fed a vitamin E-deficient torula yeast diet developed liver necrosis, hemorrhages in the gastrointestinal tract and lymph nodes, and yellowish-brown discoloration of the body fat (17, 18), conditions which could be prevented by feeding of vitamin E or of sodium selenite. Grant & Thafvelin (19) produced a similar syndrome in pigs with a vitamin E-deficient soybean oil meal diet and prevented it with 0.2 mg. of sodium selenite per kg. of diet. In addition to liver necrosis, they observed the development of massive transudates, of myocardial and skeletal muscle degeneration, and the occurrence of ceroid deposits in adipose tissues of the deficient animals; the two last-mentioned manifestations of the deficiency were not prevented by selenium. Pellegrini (20) also reported that liver necrosis and degenerative changes in cardiac and skeletal muscle of pigs fed a vitamin E-deficient torula yeast diet could be prevented by sodium selenite or by *d*-α-tocopheryl acetate, but not by addition of 0.3 per cent of L-cystine.

Welch *et al.* (21) found that feeding of sodium selenite (0.5 p.p.m. Se) to ewes, which during pregnancy received a basal ration of poor quality alfalfa hay, corn and corn cobs, and 4 per cent fish oil, appeared to decrease the severity of symptoms of white muscle disease of the lambs; when the ewes were treated with 500 mg. of α-tocopherol twice weekly during pregnancy, the lambs were normal. Proctor *et al.* (22) observed that 1 p.p.m. Se as sodium selenite, fed with a dystrophogenic diet to ewes, gave almost complete protection against muscular dystrophy of the lambs; linseed oil meal, which,

on the basis of neutron-activation analysis (23), contained about 100 times as much selenium as the kidney beans of the basal diet, also provided marked protection, as did the feeding of 100 I. U. of α-tocopheryl acetate per ewe per day. Muth *et al.* (24, 25) produced a high incidence of white muscle disease in lambs by feeding to ewes a ration of ladino-clover hay (containing less than 0.1 p.p.m. Se) and oats. Addition of 0.1 p.p.m. Se as sodium selenite prevented the development, in most of the lambs, of microscopic changes characteristic of white muscle disease and, in all lambs, of clinical signs of stiffness. Drake *et al.* (26) reported that, during a recent lambing season, serious losses from white muscle disease occurred in several areas of New Zealand. Their limited data indicate that parenteral administration of sodium selenite (1 mg. Se per lamb) was very effective in reducing the incidence of the disease. It is possible that for the prevention of this and other myopathies both selenium and vitamin E are required and that the relative amounts of each are important for the induction, severity, and symptomatology of the disease, as well as for the effectiveness of prophylactic and curative treatment with either factor. It is well established (27) that the incidence of muscular dystrophy in calves and lambs varies in different areas and that it is not always associated with a low tocopherol content of the ration, blood, colostrum, or milk of the dams.

In contrast to the effectiveness of selenium in the conditions referred to above, muscular dystrophy in rabbits could not be prevented by intramuscular injections of sodium selenite (28) or by the feeding (29) of selenium as sodium selenite (1 p.p.m.) or as DL-selenocystine (0.5 p.p.m.).

Concentrates of "Factor 3" or selenium in the form of sodium selenite (2, 6) protected chickens against exudative diathesis. In fact, Bieri & Briggs (30) have reported that, with a vitamin E-deficient diet that was devoid of other antioxidants and contained, with other components, isolated soybean protein, 4 per cent stripped lard, and 0.5 p.p.m. Se as sodium selenite, the growth and development of chickens was "normal," the same as when the diet contained 50 mg. of α-tocopheryl acetate per kg. of diet but no added selenium. No vitamin E was detactable in the eggs or tissues of the vitamin E-deficient, selenium-fed chickens. Some of their birds were kept for one year (31). It would be of interest to determine the effect of such a diet on the embryonic development, hatchability, and survival of the progeny, particularly in view of the report by Ferguson *et al.* (32) that the addition of 0.1 p.p.m. Se to a vitamin E-deficient diet of turkeys did not increase the hatchability of the eggs. It has also been mentioned (33), without documentation, that exudative diathesis in turkeys can be prevented by selenium.

Another manifestation of vitamin-E deficiency in chickens is encephalomalacia, which, in contrast to exudative diathesis (34), can be prevented by some synthetic antioxidants (see 35). It was induced (30) when the lard content of the vitamin E-deficient diet was increased from 4 to 10 or 25 per cent. Encephalomalacia could not be prevented by selenium, a conclusion previously reached by others (36, 37, 38). Bieri & Briggs (30) concluded from their

studies that "in the presence of selenium, α-tocopherol has no biochemical function in the growing chick other than that of a non-specific antioxidant."

Nesheim & Scott (39), however, have observed only partial protection against muscular dystrophy in chickens by use of amounts of sodium selenite that exceeded the amounts they found necessary for maximum growth of vitamin E-supplemented birds; vitamin E gave complete protection.

Effects of diet on the production of liver necrosis and other syndromes affected by selenium.—In their summaries of earlier work, Schwarz (40) and Goettsch (41) pointed out that the principal requirement for production of liver necrosis was, in general, a vitamin E-deficient diet that was low in cystine and usually low in protein. Some of the early work on this syndrome was done with casein diets, the necrogenic effect of which was variable, unpredictable, and, apparently, contingent on the source and method of preparation of the casein (42). Specimens of purified casein have been encountered that contained enough "Factor 3" activity to prevent exudative diathesis and encephalomalacia of chickens fed vitamin E-deficient diets (31). In later studies, diets containing torula food yeast (10, 40) or British bakers' (43) or brewers' yeast (44) have commonly been used. Amino acid analysis of different yeasts has not revealed differences in composition (45) that could be convincingly related to necrogenic properties. Bieri *et al.* (46) found that the appearance of exudative diathesis in chickens was accelerated when *torula* yeast was added to a vitamin E-deficient diet, which contained soybean protein and gelatin. This property of the yeast was associated with its ash. Salt balance may play an important role in the development of this syndrome.

Luckey *et al.* (44) have observed that "germ-free" rats raised on a vitamin E-deficient, potentially necrogenic diet did not develop liver necrosis unless their food intake was restricted to that of conventionally reared animals; this suggests that the former may have obtained sufficient selenium as a result of greater food consumption.

Diets containing soybean protein preparations have also produced liver necrosis in rats (13) and exudative diathesis in chickens (30, 39). A diet containing 30 per cent of partially purified soybean proteins was used to induce exudative diathesis in chicks and to demonstrate the prophylactic effect of selenium (30); however, a diet containing 20 per cent of solvent-extracted soybean oil meal gave complete protection against necrotic liver degeneration in rats (29). This suggests either that there is much variation in the selenium content of soybeans or that a major portion of the selenium is associated with the non-fat, non-protein portion of the soybean. On the basis of earlier evidence (47), one would expect most of the selenium in plant tissue to be concentrated in the protein fraction. In animal tissues, selenium also becomes extensively associated with proteins (48, 49).

Fink and his associates (50, 51) observed that vitamin E-deficient diets that contained 10 per cent protein, mostly in the form of dried skim milk powder, produced a high incidence of liver necrosis in rats. Not all specimens of dried skim milk had the same properties; those produced by the roller

process in general gave the highest incidence of necrosis. The syndrome could be prevented by increasing the proportion of skim milk in the deficient diet (52), by substituting fresh skim milk for the dried product (51, 53), or by the addition of traces of sodium selenite (54). It is conceivable that, during the drying, particularly at the higher temperatures of the roller process, small amounts of selenium compounds or of natural antioxidants were volatilized so that their concentration in the dried product was below the protective level when the diet contained only 10 per cent of protein. Fink (54, 55) raised the question: "Wäre es sinnvoll durch Selenzugabe die Trockenmilch vorsorglich zu vergüten?"

It is well recognized that a necrogenic diet must be vitamin E-deficient. Cystine and methionine (40, 41) were earlier found to have protective effects, but thioethanolamine or pantethine did not (56). Schwarz has suggested (7, 57) that the protective effect of cystine may be attributed to contamination with selenium compounds, whereas Yang *et al.* (14) concluded that their specimen of cystine did not contain sufficient selenium to account for its effectiveness against dietary necrotic liver degeneration. In view of the small amounts of selenium involved, the variation in selenium content of different soils (3), and its ready biological incorporation into organic compounds of plants and animals (48, 58), it is not surprising that diets containing products of natural origin and different salt mixtures, though partly purified, may produce variable results. Moreover, the effect of dietary components other than selenium and vitamin E on the complex manifestations of the deficiencies in various species remains to be established. There is an urgent need for reliable analyses of the selenium content of feeds and foodstuffs. Only few recent analyses of natural products, which are meaningful with respect to selenium deficiency, are available at the present time (29, 39, 59).

Biological activity of different selenium compounds.—The most potent source of protective activity against dietary necrotic liver degeneration of rats, which has been discovered to date, is a concentrate prepared from acid hydrolysates of hog kidney (60). It has been designated as "α-Factor 3" to differentiate it from "β-Factor 3," a chemically related but different substance present in the same hydrolysate. "α-Factor 3" is a water-soluble organic compound which, in apparently impure form, contains about 0.2 per cent of selenium (60). To obtain 50 per cent protection against liver necrosis in rats, 0.7 μg. Se per 100 gm. of diet, in the form of "α-Factor 3," was required. With many other inorganic and organic selenium compounds assayed, the amounts of selenium required for 50 per cent protection were appreciably higher, for example, about 2μg. per 100 gm. of diet for sodium selenate, sodium selenite, DL-selenocystine, selenocystathionine, and DL-selenomethionine (60). 6-Selenoctic acid, presumably a racemic mixture, was only about one-fifth as active as "α-Factor 3" (33). Some organic selenium compounds, for instance, 2-selenouracil, were inactive in the rat test (60). It appears likely that several or all of these and some other active selenium compounds serve as donors of selenium or of partial molecular structures for the bio-

synthesis of one or more substances that are responsible, *in vivo*, for the selenium effect at the molecular level. Thus, it has been demonstrated that, after administration of $^{75}SeCl_4$ to a dog, at least three different selenium compounds occur in the liver (49). In the rat, after subcutaneous injection of ^{75}Se-selenious acid, the radioactive isotope was not uniformly distributed in all fractions of homogenates of perfused livers (48); the highest concentrations, per mg. of nitrogen, were found in the microsomes and the "soluble fraction."

The studies summarized above are of great interest in regard to several interrelated aspects of animal nutrition. It is apparent that selenium compounds (at least those which have been tested thus far) cannot prevent all manifestations of vitamin-E deficiency in all species, particularly when stress factors impose more stringent dietary requirements. In addition to the failure of selenium to prevent encephalomalacia in chickens (30, 36, 37) and muscular dystrophy in rabbits (28, 29), it has also been reported (10) that, in rats fed a low-protein, vitamin E-free diet, sodium selenite merely postponed the development of liver necrosis, whereas vitamin E gave complete protection. When desiccated thyroid powder was added to a necrogenic diet (61), the development of liver necrosis was hastened and survival shortened; sodium selenite, under these conditions prolonged survival and prevented necrotic livers. Harris *et al.* (62) reported that selenium compounds cannot protect pregnant, vitamin E-deficient rats against resorption. In Dam's laboratory, it was observed that (63, 64) feeding of selenium compounds to rats did not prevent the following manifestations of vitamin-E deficiency in this species: decreased storage of vitamin A in the liver, depigmentation of incisors, peroxidation and discoloration of body fat, brown discoloration of the uterus, increased susceptibility of erythrocytes to hemolysis *in vitro*, and impaired reproductive capacity.

In addition, Corwin & Schwarz (11) found that the feeding of sodium selenite did not prevent the decline of succinate oxidation by liver slices of vitamin E-deficient rats. This phenomenon could be prevented, however, by the feeding of vitamin E or by intraportal injections of tocopherols shortly before removal of the livers (7). Moreover, the respiratory decline could also be prevented by the addition, *in vitro*, of 2-(3-hydroxy-3-methyl-5-carboxypentyl)-3,5,6-trimethyl benzoquinone or its α-lactone, both metabolites of α-tocopherol (65), the structure of which has been confirmed by synthesis (66). This observation, if confirmed, is of particular interest because substituted benzoquinones and naphthoquinones have been implicated in reactions of electron transfer (67, 68), which occur coincident with oxidation of succinate, and with oxidative phosphorylation (69, 70, 71), although the benzoquinones have no vitamin-E activity (72). Riegl *et al.* (73) have reported, however, that coenzyme Q_{10}, one of the naturally occurring benzoquinone derivatives discovered recently did not protect against dietary necrotic liver degeneration in rats.

Based primarily on studies with isooctane-treated enzyme preparations,

Nason and his associates [summarized in (74)] proposed that vitamin E plays a primary role at the enzymatic level through interaction in the electron transfer between reduced diphosphopyridine nucleotide or succinate and cytochrome-c_1 and that its function as an antioxidant is a secondary activity. Since then it has been reported, however, that the restoration of activity of the succinic-cytochrome-*c* reductase system in enzyme preparations, which had been treated with isooctane or other hydrocarbons or with bromoalkanes (75), is not specific for tocopherols; activity can also be largely restored by vitamins K_1 and K_2, by phytol or squalene (75, 76), by methyl linoleate or methyl linolenate (77), by centrifugation or freeze drying (78), or by washing of the isooctane-treated particles with sucrose solutions (79). Pollard & Bieri (79) suggested, therefore, that vitamin E has no specific role in the activity of cytochrome-*c* reductases in the intact animal body. In accord with this, Corwin & Schwarz (11) found that reduced diphosphopyridine nucleotide cytochrome-*c* reductase activity of mitochondria or microsomes from liver of rats on a necrogenic basal diet was not decreased when either vitamin E or a source of "Factor 3" was excluded from the diet. The activity of the same enzyme system was also not decreased in preparations of heart muscle from chickens, which had been fed a vitamin E-deficient diet supplemented with 0.5 p.p.m. selenium as sodium selenite for 12 weeks (79).

Failure to demonstrate thus far by decisive evidence a specific role of vitamin E at the enzymatic level tends to focus more attention on its properties as a non-specific antioxidant. The selenium compounds present in the animal organism after administration of "Factor 3" preparations or other active sources of selenium may well function in a similar manner. Dam *et al.* (37) have mentioned that feeding of 5 p.p.m. Se as selenium dioxide to chickens on a vitamin E-deficient torula yeast diet prevented not only the development of exudative diathesis but also suppressed peroxidative changes in the body fat of the birds. The feeding of synthetic antioxidants can prevent several manifestations of vitamin-E deficiency (see 80) while, in contrast, antivitamin E stress factors are related to lipid peroxides (81). Protection against dietary necrotic liver degeneration without the feeding of vitamin E or selenium compounds has been obtained (82) by the addition of some antioxidants to the diet of rats; among these, N,N′-diphenyl-*p*-phenylene diamine was most effective, giving complete protection at a level of 5 mg. per 100 gm. of diet. The same compound, which is also effective in the prevention of encephalomalacia in chickens (83) and, apparently, of muscular dystrophy of rabbits (84), did not, however, prevent muscular dystrophy in lambs (85). Inasmuch as no satisfactory correlation between antioxidant activity and protection against all manifestations of vitamin-E deficiency has been established as yet, it would be worthwhile to analyze the various antioxidants for contamination with selenium.

Further work must clarify the specific roles, if any, that can be ascribed to the tocopherols, selenium compounds, and antioxidants in terms of their need for or effect on enzyme activities as related to the gross manifestations

of vitamin-E deficiency in various species. Caution is needed in the interpretation of evidence obtained from experiments, *in vitro*, by which specific functions (86) in enzymatic processes are ascribed to tocopherols, as reflecting similar effects *in vivo*. When compounds such as the tocophorols, which have demonstrated antioxidant activity, are involved, non-specific changes can occur during the manipulation of tissues as extracts, which do not reflect the conditions *in vivo*. The formation of peroxides in such preparations, as evidenced by a positive thiobarbituric acid test, has been mentioned (31).

The Origin of Substituted Benzoquinones in Animal Tissues

With the discovery that non-saponifiable lipids in animal tissues contained quinone-like compounds (87, 88), which are also found in plants (89), yeasts (88, 90), and microorganisms (90), and that these substances are involved in oxidation-reduction reactions of the electron transport system in mitochondrial lipoproteins (91), their origin and possible nutritional significance became of interest. When these compounds, now referred to as ubiquinones or coenzymes Q, were recognized to be substituted dimethoxybenzoquinones (88, 92, 93), Morton (87) suggested that:

> Ubiquinone is in some sense a vitamin for the animal (inasmuch as the synthesis of the benzene ring cannot be effected), but until its biogenesis has been established its status in this respect remains obscure. No diet at present in use for inducing a deficiency of fat-soluble vitamins has so far interfered with ubiquinone contents.

Inasmuch as it has been possible (94) to maintain rats for three successive generations with diets assembled from compounds of known chemical structure, in which highly purified specimens of the methyl linoleate-urea complex were the only source of lipid (other than vitamins), a dietary requirement for benzoquinone derivatives for the rat is doubtful, unless microbial synthesis in the intestinal tract is assumed to be a source of such compounds. Recent evidence has clarified this problem at least in part. Gloor & Wiss (95) reported that ^{14}C-labeled mevalonic acid contributes carbon atoms to the isoprenoid side chain of the benzoquinone. They suggested that the cyclic component must be furnished to the animal and that the isoprenoid side chain can be built up partly or *in toto* from mevalonic acid. This mode of biosynthesis of the coenzymes Q would be analogous to the attachment, *in vivo*, by the rat and the chicken of a C_{20} isoprenoid side chain into 2-methyl-1,4-naphthoquinone, as reported by Martius & Esser (96). Dialemeh & Olson (97) found that a radioactive, non-saponifiable fraction isolated from the livers of rats, which had been injected with acetate-1-^{14}C, upon chromatography moved with synthetic coenzyme Q_{10}; the vitamin A fraction contained no radioactivity. Gloor & Wiss (98) isolated coenzyme Q_{10} [ubiquinone (50), according to their nomenclature (93)] from the livers of rats maintained on a vitamin A-deficient diet and injected with mevalonic acid-2-^{14}C 24 hr. prior to removal of the tissues; from 5 to 9 per cent of the radioactivity was re-

covered in the quinone, the higher yield was from the more severely vitamin-A-deficient animals. When they administered tritiated 2,3-dimethoxy-5-methyl-1,4-benzoquinone to rats, the non-saponifiable lipid fraction corresponding to coenzyme Q_{10} did not contain radioactivity. These observations indicate that either this trisubstituted benzoquinone is not a suitable precursor or that the rat can synthesize the dimethoxybenzoquinone structure. Through use of tritiated *dl*-α-tocopherol, Gloor & Wiss (98) also ruled out the conversion of the substituted chroman structure of these compounds into coenzyme Q_{10}. Full clarification of the origin of methoxybenzoquinones awaits further research. It is of interest to note, however, that they occur in fairly high concentrations in liver tissues of many different (90, 99, 100) species, including man (101); the highest value thus far reported is about 0.1 mg. per gm. in pullet livers (99).

Developments and Trends in the Nutrition of Livestock and Poultry

Progress and problems.—The fiftieth anniversary of the meeting at which the American Society of Animal Nutrition was founded occurred in November, 1958. This organization, no longer in existence, was the predecessor of what became, in 1915, the American Society of Animal Production. The November, 1958, issue of the *Journal of Animal Science* contains a series of articles in which 50 years of progress in the nutrition of beef cattle, sheep, swine, and horses and in animal physiology were reviewed by specialists (102 to 106). Nutrition of dairy cattle and of poultry was not covered in these reviews; the pertinent subject matter from American laboratories is usually reported in the *Journal of Dairy Science* or in *Poultry Science*.

The advances in the nutrition of farm animals during the last 50, particularly during the last 15, years, have been profound. Together with increased productive capacity of the animal population through selective breeding, better methods of management and sanitation, more widespread use of high-quality feed crops, such as the soybean, and more extensive and sophisticated veterinary practice, the advances in animal nutrition have brought about a great increase in the amounts of high-quality food from animal sources for use in human nutrition. Not too many years ago, most of the feed of animals was based on home-grown crops, usually mixed on the farm according to empirical experience and expediency or following some recommended formula. In the United States, home mixing of feeds is steadily declining except in the largest, highly mechanized enterprises, which operate with a great degree of technical skill.

For the feeding of ruminants, a major portion of the ration is, of course, still based on pasture, hay, and silage. But commercially mixed feeds and feed concentrates have become increasingly prominent in the nutrition of all classes of farm animals. In the last twelve years, for instance, the tonnage of feed manufactured per unit of grain-consuming animal has increased about 45 per cent in the United States (107). This development can be credited in no small measure to an aggressive and highly competitive feed-

manufacturing industry. Working in concert with agricultural experiment stations and with the chemical and pharmaceutical industries, the feed manufacturers have been quick to apply new developments in biochemistry, in fundamental aspects of animal nutrition, and in medicinal chemistry to their products. The spectacular results, in terms of improved health and productive performance of farm animals, which followed, about 35 years ago, the addition of compounds of phosphorus and iodine to natural feeds deficient in these nutrients, provided the first impetus for the improvement of feeds through the addition of chemicals. Initially, the aim of this practice was largely directed at the correction of nutritional deficiencies in feeds. Subsequently, when suitable compounds became available and their biological activities were recognized, they were introduced as extraneous additives into feeds. An important point of departure for this development was the observation by Moore *et al.* (108), in 1946, that the addition of streptomycin and some other bacteriostatic compounds to a purified diet increased the growth rate of young chickens. At about the same time, Morehouse & Mayfield (109) found that some arylarsonic acids added to the diet had not only coccidiostatic properties but that they also increased the growth rate of chickens. Residues from the production of some antibiotics were later recognized to be good sources of vitamin B_{12} and, in addition, found to have growth-promoting activities, particularly for swine and poultry (110, 111), through their residual content of antibiotic compounds. Since that time, the feed bag contains not only the nutrients needed to meet the requirements of animals for calories, proteins or amino acids, lipids, minerals, and vitamins but, in addition, a large array of compounds that serve as growth stimulants, physiological regulators, potentiators, antibiotics, and medicaments for many purposes. Judging from the flood of publications on this subject, the search for and evaluation of new and "better" feed additives continues at an accelerating pace. Interest in feed additives is growing in all livestock-producing countries, as indicated by the titles appearing in *Nutrition Abstracts and Reviews*. In many countries, this development has been approached with perhaps greater circumspection than in the United States.

The introduction of chemical additives to feeds and other changes in feeding practice have had well documented success. Hanson (105) has illustrated the results of comparative feeding tests with swine, which were fed rations compounded to simulate accepted practice in 1910, 1930, and 1953. Starting with pigs weighing about 50 lb., the animals receiving the 1953 ration reached a weight of 200 lb. in 124 days, those fed the 1930 ration reached the same weight in 190 days, while those fed the 1910 ration weighed only 122 lb. at that time. Coincident with the increased growth rate, there was also a marked decrease in the amount of feed required per pound of gain. The younger animals were more severely affected by inadequate diets than older, heavier animals.

Perry & Beeson (112) compared the performance of identical twin steers fed rations patterned after those in use in 1908 and in 1958. The animal fed

the 1958 ration showed a 45 per cent greater rate of weight gain and required 30 per cent less feed per unit of gain in weight.

Combs (113) made a similar comparison with broiler chickens. With an 1958-type experimental diet, it required 44 days and 3.12 lb. of feed to produce a 3 lb. bird; with a 1912-type ration, 78 days and 10 lb. of feed were needed. Machlin & Gordon (114) have also reported extremely rapid and efficient growth of chickens.

Concurrent with success, the use of feed additives has created new problems for the feed industry, the user, supervisory and control agencies, and the veterinary profession; these problems must be recognized without complacency. A recent report (115) has called attention to one aspect of this development:

> Change is inevitable and progress will not be stayed—in the feed business as elsewhere. But when the pace gets so fast that feed manufacturers, regulatory services and others cannot keep up, and the rapidity of change completely outstrips control methods, danger lies ahead. Sometimes a light application of brakes is not only beneficial but necessary to prevent what might become a headlong race to disaster.

A recent ruling based on an amendment of the Federal Food, Drug and Cosmetic Act (116), which provides the following:

> No additive shall be deemed to be safe if it is found to induce cancer when ingested by man or animals, or if it is found, after tests which are appropriate for evaluation of the safety of food additives, to induce cancer in animal or man.

may serve as "the application of brakes" suggested above; it may afford opportunity for careful and realistic appraisal of the whole problem. The volume of feed additives in current use is enormous. In the United States, for instance, over 500,000 lb. of antibiotics are mixed into feeds annually. The proceedings of a symposium on medicated feeds, held in 1956, contain a valuable summary of much of the work published to that time (117).

More serious than the inability of regulatory agencies to meet the demands made on them—primarily an administrative and financial problem—is the dearth of fundamental knowledge about the action of various feed additives in those species to which they are administered. Dubos (118) warned:

> There is no doubt that the equilibrium between body and microorganism can be readily upset for better or for worse as a result of the administration of drugs. As is true for any complex ecological situation, on the other hand, it is extremely difficult, if not impossible, to foresee the ultimate consequences of any intervention even when it consists in the use of a selective drug effective against only one or a few components of ecological systems.

Meager as our fundamental information is about the action of individual feed additives, we understand even less about the possible synergistic effects of these compounds, all of which are, of necessity, substances with high bio-

logical activity. There is no a priori assurance that a compound that is well tolerated by one species will not be very toxic for others. The production of fatal aplastic anemia in cattle (119) by the feeding of trichloroethylene-extracted soybean oil meal in amounts which are well tolerated by swine and poultry provided a striking example of great differences in susceptibility of different species and specific tissues to a biologically active compound.

Related to the problem of possible toxic effects of feed to animals are questions of safety of human food produced by livestock and poultry. Transmission of small amounts of the feed additives or their biologically active products of metabolism into meat, milk, and eggs has been demonstrated in some cases and led to the ban on the use of some compounds in animal feeds. For example N,N′-diphenyl-*p*-phenylene diamine, which a few years ago was used extensively as an antioxidant in broiler rations (120) and in some dairy rations, was later found to appear in eggs and in milk (121). It was also observed that this compound prolonged gestation (122) in rats, caused a very high incidence of still-born (122, 123), and increased maternal mortality (122). Its use as an additive was then prohibited.

We have then, in fact, been accepting "calculated risks" for animals and man without having a reliable basis for making calculations, a situation which, of course, is not restricted to the problem under discussion. Considering the results, many people are evidently willing to accept such risks.

Objectives of the use of additives to feeds.—The ultimate objective of the use of additives to feeds is a maximum return, in terms of usable animal products, per unit of feed. This implies the attainment of goals, not clearly defined at present, in potential biological performance of our productive animals. We do not know what the maximum possible growth rate, the maximum attainable efficiency of food utilization, the optimum "state of health," or the minimum conditions of stress may be, nor do we understand the interaction of these conditions as they are being approached. Are these goals attainable in agricultural practice where operations will be governed by economics?

The approach toward maximum productive performance of domestic animals has many facets, each of which comes into play in the consideration of additives to feeds. Even a cursory review of recent literature indicates that most of the following conditions are subject to manipulation by dietary means, including additives to feeds: increased growth rate; increased efficiency of feed utilization; increased production of meat, milk, eggs, and wool; decreased maintenance requirements; decreased requirements for specific nutrients, such as proteins and some of the vitamins; greater efficiency of digestion and absorption of nutrients; more efficient reproductive performance in terms of large litters, decreased embryonic or postnatal mortality, and shorter preweaning periods through supplementation of the diet of the young; longer productive life span; increased resistance to disease and infections; prevention and, if necessary, control of parasitic infestations; prevention of metabolic derangements under stress; stabilization of labile dietary ingredients; stabilization of labile components in products of animal

origin; and increased quality and acceptability of animal products. The opportunities for accomplishment in these areas are immense.

Current trends.—The developments in the area of additives to feeds have been summarized in several review articles and symposia (117, 124 to 129). The following examples serve to illustrate current trends and accomplishments in the use of additives to feeds.

Increased growth rates ranging from 5 up to 30 per cent (128) have been obtained by the incorporation of various antibiotics in amounts ranging generally from four to about 50 parts per million into feeds of avian and mammalian species, including young ruminants. The growth response to the antibiotics is variable, partly depending, in avian species and in swine (129), on the environmental conditions. On premises which were not previously used to house animals or were not maintained under very stringent conditions of sanitation, a growth response to antibiotic feeding may not be demonstrable; under more practical, though excellent, conditions of housing and management, antibiotics are usually effective. From such observations arose the concept that most animal populations are affected by a natural, variable, environmental "disease level" (130) or status of infection (129), which is not recognizable by clinical observations or diagnostic procedures but nevertheless of significance in affecting the growth rate of the young. This concept is supported by the observations of Luckey (131, 132) that, in contrast to conventionally reared birds, germ-free chickens showed no growth response to the feeding of bacitracin, chloramphenicol, terramycin, or procaine penicillin. Germ-free turkey poults fed procaine penicillin grew more rapidly after the initial two weeks than those on the unsupplemented diet (131). Forbes *et al.* (133) reported, however, that neither procaine penicillin or oleandomycin increased the growth of germ-free turkey poults. In an extensive study, Forbes & Park (134) found that germ-free chicks consumed more feed and gained more weight than birds reared in a non-sterile or deliberately infected environment. They concluded that one or more components of the intestinal flora have a growth-depressing effect, which can be overcome by feeding of penicillin salts. Lev & Forbes (135) also observed no growth response of "germ-free" chickens to feeding of procaine penicillin. The growth rate of "germ-free" chickens was not depressed when an intestinal flora of *Escherichia coli*, *Lactobacillus lactis*, or *Streptococcus liquefaciens*, singly or in combination, was established; when a flora of *Clostridum welchii* was introduced, however, the antibiotic did improve the growth of the birds. In this connection, the work of Barnes *et al.* (136) is pertinent. They found that the growth of rats fed diets containing sub-optimal amounts of thiamine was increased by addition of penicillin to the diet only when coprophagy was prevented.

One important practical consequence of the addition of antibiotics to feeds, particularly in the swine industry, is the great reduction in the number of unthrifty animals, runts, which grow slowly. Such animals have shown spectacular responses to feeds containing small amounts of antibiotics (137).

A variable disease level seems to prevail not only in herds or flocks in a given environment but also in individuals.

Several recent reports (138, 139, 140) have confirmed earlier observations (141, 142) that feeding of relatively large amounts, 40 to 100 mg. per day, of chlortetracycline to young dairy calves can increase their growth rates by as much as 20 to 30 per cent. One of the major benefits was a reduced incidence of scouring (138). It has been suggested (140) that antibiotic supplementation in calves involves metabolic and endocrine effects not a reduction of the disease level.

As new antibiotics are discovered, which meet the requirements of the Food and Drug Administration, and become available at competitive costs, they will no doubt be tried as feed additives, partly to replace others which may loose their effectiveness (143, 144).

Implants of estrogens, mainly diethylstilbestrol and related stilbene derivatives, have been used for several years to improve growth, efficiency of feed utilization, and fattening of poultry (145) and, with varying success, also of steers and lambs (146, 147). Burroughs *et al.* (148) reported that daily oral administration of 5 mg. diethylstilbestrol to steers increased weight gains by as much as 35 per cent and reduced feed costs. It has been estimated (149) that now about 70 per cent of the mixed feeds for beef cattle contain diethylstilbestrol. With lambs, good results have also been reported, but carcass quality is frequently depressed (147). With swine (149, 150) and poultry (151), oral feeding of synthetic estrogens has little or no growth-promoting effect. On the basis of limited trials (152, 153), it was reported that feeding of gibberellins promoted growth in steers and lambs.

Tranquilizers have invaded the feed bag. Sherman *et al.* (154) reported growth promotion in steers and lambs by adding tranquilizers to feed at levels that did not produce sedation. Others (147, 155) observed no growth response with lambs. With poultry, the results of various studies are also not in accord. Some investigators (156, 157) reported favorable, others none (158, 159) or unfavorable (158, 160) effects, depending partly on dosage. Much work is in progress with tranquilizers, but it will require many trials with the available compounds before their general usefulness, limitations, proper dosage, and safety can be ascertained.

After earlier, generally disappointing trials with synthetic goitrogens, such as thiourea and thiouracil [reviewed by Winchester & Andrews (150); Blaxter *et al.* (161); Hill (162); Sykes (163)], renewed interest in such compounds was stimulated by the reports of Burroughs *et al.* (164, 165) that feeding of 600 mg. of 1-methyl-2-mercaptoimidazole per day to steers increased their weight gains by 22 per cent and the efficiency of feed utilization by 13 per cent. Blair *et al.* (166) found that this compound promoted growth only if fed to lambs that also carried implants of diethylstilbestrol. They observed no improvement in the coefficients of digestibility of the major components of the feed.

Iodinated casein is still in use as a feed additive, mainly in rations of dairy cattle. Its effectiveness and limitations have been reviewed by Blaxter

et al. (161), by Moore (167), and in a publication of the National Research Council (168).

Several organic arsenicals, such as 3-nitro-4-hydroxybenzene arsonic acid, continue to be used as growth promoters for non-ruminants and as coccidiostats for birds (169). The effective dosage, generally 0.005 to 0.01 per cent of the mixed feed, leaves only a small residue of arsenic in the tissues, which disappears within a few days after feeding of the drug has ceased (169).

Effect of additives on utilization of nutrients.—In most instances, the promotion of growth by feed additives is associated with greater efficiency of feed utilization. The reasons for this economically important phenomenon are not entirely clear; one lies in greater feed consumption, as a consequence of which a smaller proportion of the absorbed nutrients is needed to meet the maintenance requirements. Burch & Hunter (170) studied oxidative phosphorylation of β-hydroxybutyrate in mitochondria from livers of normal and riboflavin-deficient rats; they suggested that the deficient animals utilized their feed less efficiently because of some difficulty in oxidative phosphorylation.

The mechanisms by which various feed additives produce their effects are not well known. In the case of the antibiotics, various theories and their supporting evidence were summarized in the "Proceedings of the First International Conference on the Use of Antibiotics in Agriculture" (127). Although systemic effects have not been ruled out, most of the evidence centers on interrelationships between the host and the intestinal flora with respect to synthesis, and absorption or destruction of essential nutrients or of toxic products and on the morphological changes in the intestinal tract. Hock *et al.* (171) attempted to correlate respiratory activity of the intestinal mucosa of chicks with administration of chlortetracycline. In young birds, they found that its addition to feed did not depress the Q_{O_2} of the mucosa; in birds older than 30 days, a significant depression was reported. Bush *et al.* (172) found that feeding of chlortetracycline to dairy calves, while promoting growth, had no effect on the apparent digestibility of cellulose [see also (173)], crude protein, or dry matter, on the retention of nitrogen and ash, or on the utilization of calcium. Sewell & Keen (174) reported that there was a greater growth response of pigs to the feeding of chlortetracycline at lower than at higher protein levels; they observed no significant interaction between protein level and antibiotic. This is in line with the earlier data of Forbes (175), who found that, in young male rats, streptomycin and chloramphenicol increased the apparent and true digestibility and the retention of nitrogen, while decreasing endogenous nitrogen excretion. Feeding of estrogens was also reported (176) to decrease the protein requirements of turkeys.

At present, there is much interest [Fritz (177); Jensen (178)] in the use of fungal and bacterial enzymes either as additives to feeds or for predigestion of certain feeds. Not enough reliable evidence has been published to permit evaluation of the general usefulness of such practice. With chickens fed an enzyme-treated barley ration (179) or with steers fed rations contain-

ing low-moisture corn with added enzymes (180), promising results have been reported. With swine, treatment of a barley ration with amylolytic enzyme did not increase weight gains appreciably (181).

The absorption of nutrients from the intestine can be altered by additives to the diet. Draper (182) observed that lysine-^{14}C disappeared more rapidly from the intestine of chicks fed penicillin, implying that it was more readily absorbed, than from chicks fed without antibiotic. In rats, the absorption of vitamin B_{12} was about doubled by the addition of D-sorbitol or glucosamine hydrochloride to the diet (183). D-Sorbitol has also been reported (184, 185) to enhance the absorption of vitamin B_{12} in humans. Terephthalic acid caused a marked increase in the therapeutic effects of oxytetracycline and of erythromycin by increasing their concentration in the blood (186). This compound, however, caused a great decrease in the absorption of iron in the rat (183).

Other additives to feeds, which are under investigation, are ethanol, in combination with molasses and urea, for beef cattle (187) and lambs (188) and antioxidants for stabilization of feed components, such as carotene and fats, and for possible stabilization of edible animal fats (189).

Discussion of the large number of chemicals (117) that are administered with feeds for therapeutic or curative purposes is beyond the scope of this review.

Inadvertent Production of Disease in Animals

Several striking examples are on record, which demonstrate that through the introduction of processed components into feeds or through alterations in the formulas of mixed feeds, unexpected diseases may be induced in livestock and poultry. Before the etiology of such disease was recognized and corrective measures could be taken, serious losses had occurred. During the last ten years, for instance, there occurred outbreaks of aplastic anemia in cattle through the feeding of trichloroethylene-extracted soybean oil meal (190), of hyperkeratosis (X-disease) in cattle through the use of chlorinated naphthalenes in processing machinery (191), and of vitamin-E deficiency following the introduction of high energy rations into the broiler industry (192).

The most recent instance of this type is the development of chicken edema, or hydropericardial disease, through a dietary component. In the fall of 1957, in the flocks of poultry producers in about 10 states, what appeared to be an epidemic outbreak of a fatal disease occurred in young chicks (193); it was reported (194) to have caused the death of several million birds. The condition was characterized by poor weight gains and sudden deaths of the birds in from three to six weeks. Gross and microscopic examinations at necropsy revealed edema, ascites, accumulation of sterile (195) fluid in the pericardial sac, necrotic changes in the liver, enlarged kidneys and extensive hypertrophy, and proliferation of the endothelial cells of the vascular system (196, 197). All birds had consumed rations that contained processed animal

fat. In pullets, feeding of such rations caused delayed and decreased egg production and a high mortality of the birds; the hatchability of eggs was reduced (198). Older hens ceased laying, but their mortality was lower. The disease was not transmissible (195); it could not be prevented by feeding of *dl*-α-tocopherol (195, 200), nor could it be induced in ducks, turkeys, rats, swine, or calves with rations containing the same ingredients (199). Toxic effects from the fat component of the ration were suspected early (193), and they were traced (198, 201) to specific lots of feed-grade animal fats, which included a residue from the manufacture of higher fatty acids.

With the aid of bioassays (202, 203), it was shown that the toxic factor was a non-saponifiable lipid, not a peroxide (200). It has been concentrated about 3000-fold (201). Although not isolated in pure form, the physical and chemical properties indicate that the toxic factor contains one or more cyclic hydrocarbons (202), which have spectral properties of alkyl naphthalenes (201), and at least one aromatic ring (203). It may be derived from cholesterol during the commercial heat-processing of the fat from which it is formed (202).

A syndrome, of unknown etiology, referred to as aplastic anemia or hemorrhagic disease in chickens, is characterized by sporadic appearance and is troublesome to the broiler industry (204 to 206). It is not known whether or not feed additives or newer methods of feed formulation are in any way related to this disease or to the syndrome of dissecting aortic aneurism observed in turkeys (207).

These interesting conditions merit the attention of biochemists. They may be corrected from a practical standpoint by abandoning certain practices of feeding or management. But such problems are not solved unless the causative factors are identified and the mechanism of their action is understood. Compounds with interesting and potentially useful properties may be discovered by such investigations.

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WATER-SOLUBLE VITAMINS, PART I[1,2,3]

(ASCORBIC ACID, NICOTINIC ACID, VITAMIN B_6, BIOTIN, INOSITOL)

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This review covers the pertinent literature available to the authors up to November, 1959. Because of the large number of publications, coverage of every paper could not be given. The authors have placed particular emphasis on those papers concerned with the metabolism of the vitamins and their function at the molecular level.

L-ASCORBIC ACID

Synthesis of L-ascorbic acid in animals.—Studies in recent years have pointed out the mechanism of L-ascorbic acid synthesis in animals through the glucuronic acid pathway[4] (Figure 1). According to this scheme, D-glucose is oxidized to D-glucuronic acid which undergoes reduction to L-gulonic acid; the latter acid then serves as the precursor of both L-ascorbic acid and L-xylulose. L-Xylulose is further metabolized back to D-glucose through D-xylulose and the pentose phosphate pathway. Man, monkey, and guinea pig can carry out the various steps in the glucuronic acid pathway with the exception of the conversion of L-gulonolactone to L-ascorbic acid. The reactions of the glucuronic acid pathway have been reviewed during the past year by Touster (1) and Burns (2).

The most likely mechanism for the conversion of D-glucose to D-glucuronic acid is through the intermediate formation of UDP-glucose and UDP-glucuronic acid. Enzymes that hydrolyze UDP-glucuronic acid to D-glucuronic acid through D-glucuronic acid-1-phosphate have been reported in the kidney by Ginsburg *et al.* (3) and in the liver by Evans *et al.* (4). Further studies (4) demonstrated the overall conversion of D-galactose-1-C^{14} to labeled D-glucuronic acid and L-gulonic acid in a rat liver homogenate system fortified with ATP, UDP-glucose, and DPN. Results of *in vivo* experiments (4) in rats indicated that D-galactose-1-C^{14} was a considerably better pre-

[1] The survey of the literature pertaining to this review was concluded in November, 1959.

[2] The following abbreviations are used: CDP for cytidine diphosphate; CMP for cytidine monophosphate; CoA for coenzyme A; CTP for cytidine triphosphate; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); TPNH for triphosphopyridine nucleotide (reduced form).

[3] The authors are grateful to Miss Carole Evans for her valuable assistance in the preparation of this review.

[4] Referred to also as the glucuronic-xylulose pathway, the C-6 oxidation pathway, and the uronic acid pathway.

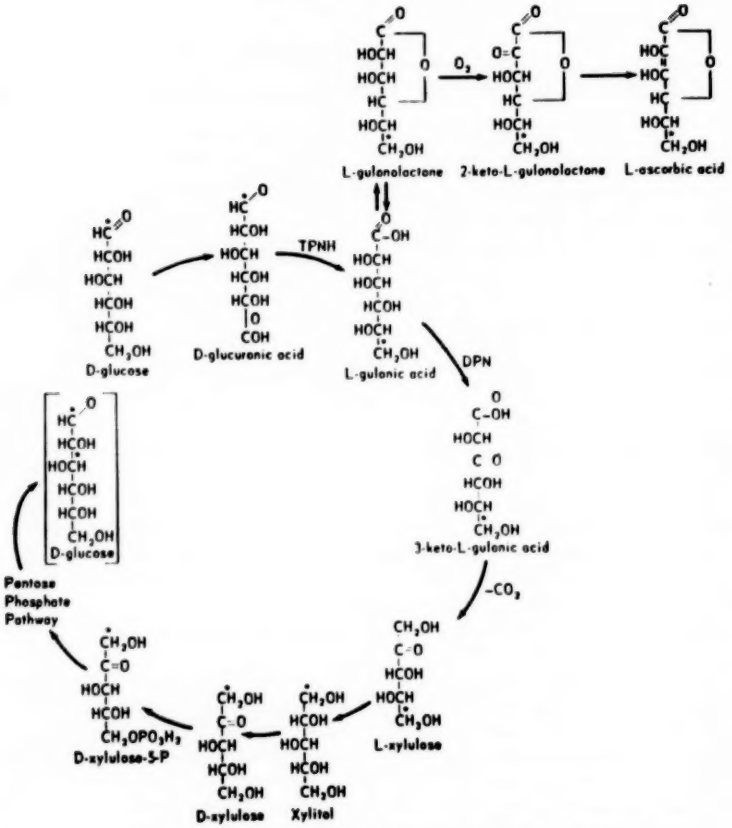

FIG. 1. Glucuronic acid pathway of glucose metabolism. The asterisks denote the fate of C-1 of glucose in this scheme

cursor of L-ascorbic acid than was D-glucose-1-C^{14}. In view of the recent studies by Siu & Wood (5) which indicated that D-galactose was a more direct precursor of UDP-glucose than was D-glucose, the results of Evans *et al.* (4) are in accord with the biosynthesis of L-ascorbic acid through the uridine nucleotides.

Winkelman & Lehninger (6) have described the properties and distribution of two lactonases which hydrolyze D-glucuronolactone and L-gulonolactone, respectively. Uronolactonase, found in the microsomes of rat liver but not in the soluble fraction, hydrolyzes D-glucuronolactone but is inactive

against a wide variety of aldonolactones. Aldonolactonase, found in the soluble fraction of rat liver but not in the microsomes, hydrolyzes L-gulonolactone and a wide variety of 5-, 6-, and 7-carbon aldonolactones. More recently, Yamada (7, 8) studied two similar lactonases in rat liver and found that they catalyzed the formation of D-glucuronolactone and L-gulonolactone from the respective acids. Chatterjee *et al.* (9) also reported the presence of lactonase activity in the soluble fraction of rat liver required for the conversion of L-gulonic acid to L-gulonolactone (Figure 1).

Mano *et al.* (10) have demonstrated the formation of L-gulonolactone from D-glucuronolactone by the action of a TPNH-dependent enzyme, which was previously reported to catalyze the reduction of D-glucuronic acid (11, 12). The enzyme was present in the soluble fraction of rat liver, and it was purified 350- to 500-fold. The purified enzyme catalyzed the reduction of D-glucuronolactone or D-glucuronic acid by TPNH in spite of the complete absence of the lactonase. Thus, the direct conversion of D-glucuronolactone to L-gulonolactone in animals is possible as well as the conversion of D-glucuronic acid to L-gulonic acid, as shown in Figure 1.

Grollman & Lehninger (13) propose that L-ascorbic acid and L-xylulose may share the same biosynthetic pathway from L-gulonic acid through the common intermediate, 3-keto-L-gulonic acid. According to this mechanism, 3-keto-L-gulonic acid would yield L-xylulose upon decarboxylation and L-ascorbic acid upon enolization and lactonization. Recent studies by Ashwell *et al.* (14), employing a purified preparation of L-gulonic acid (DPN) dehydrogenase from mammalian kidney extracts, have furnished support for 3-keto-L-gulonic acid as an intermediate in the formation of L-xylulose. However, present data are not in accord with 3-keto-L-gulonic acid as an intermediate in L-ascorbic acid biosynthesis. A number of workers (9, 15 to 18) have shown that L-gulonolactone is the direct precursor of L-ascorbic acid and that the reaction occurs in rat liver microsomes. Kanfer *et al.* (18) and Chatterjee *et al.* (19, 20) solubilized the microsomal enzyme system required for the conversion of L-gulonolactone to L-ascorbic acid, and both groups have presented evidence for 2-keto-L-gulonolactone as an intermediate in this reaction. The L-ascorbic acid-synthesizing system in liver microsomes differs from the L-xylulose-forming system in kidney extracts as follows: (*a*) L-ascorbic acid synthesis has an absolute requirement for the lactone ring, whereas L-xylulose formation requires the free acid; (*b*) L-xylulose synthesis has an obligatory requirement for DPN, while L-ascorbic acid formation is independent of the presence of pyridine nucleotides; (*c*) the synthesis of L-xylulose is accompanied by the formation of 3-keto-L-gulonic acid, whereas 2-keto-L-gulonolactone appears to be the intermediate in L-ascorbic acid biosynthesis.

Roy & Guha (21) have confirmed the earlier studies of Grollman & Lehninger (13) that L-ascorbic acid is synthesized in the kidney of birds, reptiles, and amphibia instead of in the liver as in most mammals. Roy & Guha (21) found that neither the liver nor the kidney of the red-vented bul-

bul and of the Indian fruit bat had the capacity to synthesize the vitamin. In a subsequent study, Roy & Guha (22) placed red-vented bulbuls on a scorbutic diet and produced a condition resembling scurvy, which was cured upon the administration of L-ascorbic acid. Tissue concentrations of L-ascorbic acid were not measured in these experiments.

Caputto *et al.* (23) observed that liver homogenates of tocopherol-deficient rats and rabbits synthesized L-ascorbic acid from D-glucuronolactone at 10 to 30 per cent of the rate obtained with liver homogenates from tocopherol-sufficient animals. Reactivation of L-ascorbic acid synthesis by liver extracts of deficient animals was obtained by addition of either Mn^{++} or Co^{++} salts and with ethylenediamine tetraacetic acid. More recently, this group reported (24) the successful reactivation of L-ascorbic acid synthesis from D-glucuronolactone in liver homogenates of tocopherol-deficient rats by the addition of tocopherol. Further studies by Kitabchi *et al.* (25) suggested that the site of inhibition of L-ascorbic acid synthesis in liver of vitamin E-deficient rats was in the conversion of L-gulonolactone to L-ascorbic acid by the microsomes.

Minesita *et al.* (26) reported that the administration to rats of the alkaloid lycorine interfered with L-ascorbic acid synthesis and decreased the L-ascorbic acid content of various organs, producing a condition resembling scurvy. The mechanism by which lycorine exerts this effect is not known.

A variety of drugs have been reported by Longenecker, *et al.* (27) and Conney & Burns (28) to stimulate L-ascorbic acid synthesis in the rat. These compounds included the hypnotics, chlorobutanol (Chloretone) and barbital; the analgesics, aminopyrine and antipyrine; the muscular relaxants, orphenadrine and meprobamate; the antirheumatic agent, phenylbutazone; and the carcinogenic hydrocarbons, 3-methylcholanthrene, 3,4-benzpyrene, and 1,2,5,6-dibenzanthracene. The way by which drugs exert this effect is not known, but available evidence indicates that these drugs act by stimulating the synthesis of D-glucuronic acid by a mechanism that does not involve glucuronide formation. Drugs that stimulate the synthesis of L-ascorbic acid have also been shown to increase the activity of liver microsomal enzymes which metabolize various foreign compounds (28). This stimulatory effect of drugs on L-ascorbic acid biosynthesis and on the liver microsomal enzymes appears to represent an adaptive response to foreign compounds.

Synthesis of L-*ascorbic acid in plants.*—Isherwood *et al.* (29) showed originally that D-glucuronolactone and L-gulonolactone were converted to L-ascorbic acid in cress seedlings, and they proposed a pathway for the synthesis of L-ascorbic acid in plants similar to that observed in animals. Confirmation of their results has come from recent isotopic studies of Loewus and co-workers (30, 31), which demonstrate the conversion of D-glucuronolactone to L-gulonic acid and to L-ascorbic acid in the detached ripening strawberry. Other studies of Loewus (30), however, indicate that the scheme proposed by Isherwood *et al.* (29) cannot be the major pathway for L-ascorbic acid synthesis from D-glucose in plants. When tracer methods employing

D-glucose-1-C^{14} or D-glucose-6-C^{14} were applied to a study of L-ascorbic acid formation in detached ripening strawberries and in germinating cress seedlings, no evidence was found for an inversion between D-glucose and L-ascorbic acid. Based on these results, Loewus *et al.* (30) have postulated that L-ascorbic acid may be formed primarily in plants from D-glucose-6-phosphate through the hexose monophosphate pathway instead of through the glucuronic acid pathway. The significance of such a mechanism for L-ascorbic acid synthesis in plants, however, must await information on the enzymatic steps involved.

Catabolism of L-*ascorbic acid.*—Information has appeared recently on the mechanism of L-ascorbic acid catabolism in animals. Chan *et al.* (32) showed that dehydroascorbic acid was decarboxylated in guinea pig liver homogenate with the formation of L-xylose. Burns *et al.* (33) described a system in rat kidney, which decarboxylated L-ascorbic acid through the intermediate formation of dehydroascorbic acid and diketogulonic acid, and subsequent studies by Kanfer *et al.* (34) have identified L-lyxonic acid and L-xylonic acid as products of this reaction.

Dayton *et al.* (35) have carried out *in vivo* experiments in guinea pigs with C-1- and C-6-labeled L-ascorbic acid, dehydroascorbic acid, and diketogulonic acid. The incorporation of C^{14} in expired CO_2, urinary oxalate, and liver glycogen was measured, and the results obtained are in accord with a pathway for the metabolism of the vitamin via dehydroascorbic acid and diketogulonic acid. Diketogulonic acid-6-C^{14} yielded glycogen, and the major fraction of the total C^{14} in the glucose residues was about equally distributed between C-1 and C-6. This isotopic pattern in glycogen is markedly different from that obtained in similar experiments by Eisenberg *et al.* (36) with C-6-labeled L-gulonolactone. Thus, these findings furnish evidence against any appreciable metabolism of L-ascorbic acid through L-xylulose such as is given for L-gulonolactone in Figure 1.

Loewus *et al.* (30) have carried out an experiment with L-ascorbic acid-6-C^{14} in a ripening strawberry. They observed an isotopic pattern in glucose derived from sucrose of the berry similar to that obtained in glycogen isolated from rats injected with diketogulonic acid-6-C^{14} (35). These findings suggest the possibility of similar pathways for L-ascorbic acid catabolism in plants and animals.

Functions of L-*ascorbic acid.*—Reviews by Mapson (37) and Knox (38) have appeared recently on the mechanism of action of L-ascorbic acid in animals and plants. L-Ascorbic acid has been implicated in tyrosine metabolism at the step that involves the oxidation of *p*-hydroxyphenylpyruvic acid to homogentisic acid. Zannoni & La Du (39) have recently carried out a detailed study on this particular reaction, employing a purified preparation of *p*-hydroxyphenylpyruvic acid oxidase. L-Ascorbic acid prevented the substrate-induced inhibition of *p*-hydroxyphenylpyruvic acid oxidase. The vitamin appeared to act in a non-specific manner, since reduced 2,6-dichlorophenolindophenol was considerably more effective than was L-ascorbic acid

in overcoming this inhibition. Zannoni & La Du (40) further showed that livers from scorbutic guinea pigs had about the same *p*-hydroxyphenylpyruvic acid oxidase activity as livers from normal animals. When scorbutic guinea pigs were injected intraperitoneally with *p*-hydroxyphenylpyruvic acid, the activity of liver *p*-hydroxyphenylpyruvic acid oxidase was markedly inhibited one hour after injection. The inactive enzyme was reactivated *in vitro* by the addition of either L-ascorbic acid or, more effectively, by reduced 2,6-dichlorophenolindophenol. The results suggested that the vitamin may maintain normal tyrosine metabolism by protecting *p*-hydroxyphenylpyruvic acid oxidase from inhibition by its substrate.

Kersten, *et al.* (41) and Krisch & Staudinger (42) have reported the presence in pig adrenal microsomes of a cyanide-insensitive respiratory chain. This system involved reduced DPN and cytochrome-b_5 and was stimulated by the addition of catalytic amounts of L-ascorbic acid. Evidence was presented that the L-ascorbic acid-monodehydroascorbic acid system may function in electron transport between DPNH and cytochrome-b_5 (Figure 2).

Marré & Arrigoni (43) have continued their studies on the role of L-ascorbic acid in photosynthesis. An enzyme system was extracted from the acetone powder of spinach chloroplasts, which catalyzed the oxidation of DPNH and TPNH in the presence of L-ascorbic acid and an ascorbic acid-oxidizing agent such as Cu^{++} or ascorbic acid oxidase. This system was unable to reduce dehydroascorbic acid and closely corresponds to the reduced pyridine nucleotide-monodehydroascorbic acid reductase previously shown to occur in plants. The possibility was suggested that this system may function in oxidative phosphorylation reactions of illuminated chloroplasts.

Although a need for L-ascorbic acid in collagen formation has been known, the specific biochemical mechanisms involved are still not clear. Studies of

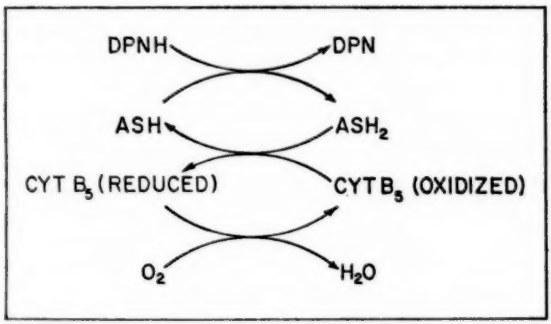

FIG. 2. Scheme for electron transport in adrenal microsomes adapted from Krisch & Staudinger (42).

AsH_2 = ascorbic acid

AsH = monodehydroascorbic acid

the carrageenin-induced granuloma in normal and scorbutic guinea pigs have led Robertson *et al.* (44) to postulate that L-ascorbic acid may be required for the formation and the incorporation of an "active" hydroxyproline into collagen. Robertson & Schwartz (45) had originally proposed a different mechanism in which the vitamin was required for the formation of hydroxyproline from bound proline in a pre-collagen peptide. Mitoma & Smith (46) have presented evidence that the hydroxylation of proline was not decreased by L-ascorbic acid deficiency in guinea pigs and that L-ascorbic acid may exert its effect on collagen synthesis indirectly by its participation in the maturation of fibroblasts. Gross (47) has postulated that a deficiency of L-ascorbic acid in guinea pigs either interferes with the synthesis of new collagen or results in its destruction as it is produced.

Changes in dental structures are a sensitive index of vitamin-C deficiency in guinea pigs. Hunt & Paynter (48) studied the teeth and jaws of guinea pigs fed a basal semisynthetic diet supplemented with low levels of L-ascorbic acid. In animals fed 5.0 mg. L-ascorbic acid per day, no microscopic alterations were observed, but on a diet of 0.4 mg. L-ascorbic acid for 75 days, severe alterations in the structure of dentine was observed, and mild alterations occurred in the normal pattern of bone deposition and resorption in the jaws. Animals kept on a vitamin-C-free diet showed the most severe alterations from normal. Hemorrhage occurred in both the pulp and periodontal membrane, and there was a failure in the formation of collagen fibers, cementum, dentine, and bone. Yale *et al.* (49) have studied the incorporation of L-ascorbic acid-1-C^{14} into the oral structures of normal and scorbutic guinea pigs. Localization of C^{14} was observed in the peridontal membrane and pulp but not in the enamel and dentine. They reported evidence for C^{14}-labeled compounds other than L-ascorbic acid, but these were not identified.

Burns *et al.* (50) have re-evaluated the vitamin-C activity of D-ascorbic acid in view of their tracer studies, which showed that D-ascorbic acid was retained by scorbutic guinea pigs to a considerably smaller extent than was L-ascorbic acid. When bioassay experiments were carried out in guinea pigs under conditions in which essentially comparable tissue concentrations of D-ascorbic acid and L-ascorbic acid were maintained, D-ascorbic acid was able to replace some of the activities of L-ascorbic acid. Weight and survival of scorbutic guinea pigs were maintained with D-ascorbic acid, but hemorrhages about the joints were not prevented. The dentine produced in animals receiving D-ascorbic acid was normal in morphology and staining properties. However, the vitamin-C effect of D-ascorbic acid was not complete in that the predentine formed in these animals was still abnormal in its reactivity to acid mucopolysaccharide stain.

Rosenberg & Culik (51) have reported that α-lipoic acid diminished symptoms of scurvy in guinea pigs on a vitamin C-free diet or in guinea pigs receiving a small suboptimal amount of L-ascorbic acid. The extent of vitamin-C activity of α-lipoic acid was evaluated from the rate of survival of scorbutic guinea pigs and from post-mortem examination. α-Lipoic acid was

also observed in these studies to have a beneficial effect in rats on a vitamin E-deficient diet. The efficacy of α-lipoic acid in alleviating symptoms of L-ascorbic acid and tocopherol deficiency was explained on the basis of a possible protective action exerted by α-lipoic acid or its dihydroderivative on the two vitamins.

Banerjee & Kawishwar (52) have studied the effect of scurvy on the tricarboxylic acid cycle. They found that citric acid and malic acid excretions were increased in scorbutic guinea pigs after the feeding of butyrate, but these excretion values diminished when the animals were treated with insulin. Banerjee *et al.* (53) have further observed decreased activities of succinic, malic, and lactic dehydrogenase in scurvy, and the activities of these enzymes were restored to normal after prolonged treatment of the guinea pigs with insulin. How L-ascorbic acid deficiency produces these effects is not clear.

Vitamin B_6

Snell (54) has recently summarized the various kinds of enzymatic reactions that require vitamin B_6 as a coenzyme. He points out that an understanding of the role of pyridoxal in enzymatic reactions has come from studies with non-enzymatic model systems involving the chelation of pyridoxal with an amino acid and a metal to form a Schiff base. In addition to the use of these model systems, antagonists of vitamin B_6 have been extensively employed to elucidate the mechanism of action of pyridoxal phosphate in biological systems. These antagonists are classified in two categories: carbonyl reagents and structural analogues of vitamin B_6.

Vitamin-B_6 antagonists.—The administration to animals of various carbonyl reagents, such as isonicotinic acid hydrazide and semicarbazide, produce convulsive seizures, presumably by inhibiting certain enzymes in brain. Killam & Bain (55) showed that carbonyl reagents inhibited the pyridoxal phosphate-requiring enzyme, glutamic decarboxylase, from rat brain, which catalyzes the conversion of glutamic acid to γ-aminobutyric acid. The decreased activity of this enzyme was correlated with decreased levels of γ-aminobutyrate in brain. McCormick & Snell (56) have proposed another mechanism for the action of these carbonyl reagents on brain metabolism. They have isolated pyridoxal phosphokinase from human cerebral cortex and purified the enzyme 200-fold. Both α-methylphenylethylhydrazine and isonicotinic acid hydrazide were found to be extremely effective inhibitors of this kinase. The actual inhibitors, however, were the corresponding hydrazones formed between these hydrazines and pyridoxal. The oxime, hydrazone, and semicarbazone of pyridoxal were all similarly potent inhibitors with affinities for the kinase well over 100 times that of pyridoxal. In view of the low concentration at which the carbonyl reagents inhibited pyridoxal phosphokinase, McCormick & Snell (56) suggested that the physiological effects of these compounds resulted in part from their capacity to lower or to eliminate production of pyridoxal phosphate. Thus, the carbonyl reagents were postulated to lower brain γ-aminobutyrate levels as a consequence of

inhibition of the pyridoxal kinase rather than from inhibition of the glutamic decarboxylase. Results of preliminary experiments by these investigators showed that glutamic decarboxylase of beef brain required almost 1000-fold greater concentration of carbonyl reagents for inhibition than did the pyridoxal kinase of this tissue.

Baxter & Roberts (57) have studied the vitamin B_6-dependent γ-aminobutyric acid–α-ketoglutaric acid transaminase of beef brain and have found that the enzyme was markedly inhibited by hydroxylamine. These investigators have also reported that administration of hydroxylamine resulted in increased γ-aminobutyrate levels in rat brain (58). Eidelberg *et al.* (59) found that administration of hydroxylamine to cats elevated brain levels of γ-aminobutyrate and reduced the duration of electrically induced seizures. The possibility was suggested that the increased γ-aminobutyrate levels may result from a more potent inhibitory effect of hydroxylamine on the γ-aminobutyric acid transaminase than on the glutamic acid decarboxylase.

Rindi & Ferrari (60) showed that the seizure-producing compound, toxopyrimidine (2-methyl-4-amino-5-hydroxymethyl pyrimidine), decreased the level of γ-aminobutyrate in rat brain, presumably by inhibition of glutamic decarboxylase. Nishizawa *et al.* (61) administered toxopyrimidine and hydroxylamine to mice and measured both the activity of brain glutamic-aspartic transaminase and glutamic decarboxylase. When convulsions were induced by toxopyrimidine administration, both enzyme systems were inhibited. When sufficient hydroxylamine was given to cause convulsions, the decarboxylase was inhibited, but the transaminase was not affected unless lethal doses of hydroxylamine were administered. These investigators suggested that the convulsions were related to inhibition of glutamic decarboxylase.

Jenkins *et al.* (62) prepared and purified glutamic-aspartic transaminase from pig heart and showed that two molecules of pyridoxal phosphate were tightly bound to each molecule of apoenzyme. Jenkins *et al.* (63) have carried out further kinetic and spectroscopic studies on the mechanism of inhibition of the purified glutamic-aspartic transaminase by isonicotinic acid hydrazide. This carbonyl reagent reacted rapidly with the pyridoxal form of the purified enzyme to yield spectrally distinct complexes. The interaction, as measured spectroscopically, was correlated quantitatively with the inhibition of the enzyme and was not caused by resolution of the holoenzyme. Two types of inhibition were observed: one was rapid and readily reversible, the other was slow and not reversible.

Gershoff & Faragalla (64) have shown that the urinary excretion of oxalate was about doubled in rats fed a vitamin B_6-deficient diet. Administration of deoxypyridoxine and isonicotinic acid hydrazide further increased oxalate excretion in these deficient animals. The possibility was suggested that the enhanced oxalate formation may result from abnormal glycine metabolism in vitamin-B_6 deficiency.

Several studies have been carried out with vitamin-B_6 inhibitors in

microorganisms. Sakuragi & Kummerow (65) have grown B_6-requiring yeast cells in the presence of various vitamin-B_6 antagonists to obtain information on the mechanism of inhibition. Four resistant strains of *Saccharomyces calsbergensis* grew well in the presence of 4-deoxypyridoxine, 4,5-bisdeoxypyridoxine, 2-methyl-4-amino-5-ethoxymethyl pyrimidine, or isonicotinic acid hydrazide. Haughton & King (66) showed that toxopyrimidine phosphate failed to inhibit several bacterial enzyme systems which require pyridoxal phosphate. These included unresolved arginine and glutamic acid decarboxylase and tryptophanase and glutamate-alanine transaminase. Yamada *et al.* (67) reported an inhibitory effect of pituitary growth hormone on tryptophanase and glutamic acid decarboxylase derived from cell-free extracts of *Escherichia coli*. The inhibition was reversed by relatively high concentrations of pyridoxal phosphate.

Vitamin-B_6 enzymes.—Saran (68) prepared kynureninase from *Neurospora crassa*, with an eightfold purification. The enzyme contained functional sulfhydryl groups and was activated by Ca^{++} and Mn^{++} ions, pyridoxal phosphate, and, to a lesser extent, by pyridoxamine phosphate. Scott & Jakoby (69) have purified γ-aminobutyric-glutamic transaminase from a strain of *Pseudomonas fluorescens*. Although no requirement for pyridoxal phosphate was shown, the enzyme may contain pyridoxal phosphate in a bound form, since inhibition was observed with carbonyl reagents, such as cyanide and hydroxylamine. Jakoby & Scott (70) have developed a sensitive and specific method for the assay of γ-aminobutyrate or α-ketoglutarate, which employs γ-aminobutyric-glutamic transaminase in a coupled reaction with the TPN-dependent succinic semialdehyde dehydrogenase. This method has been used for measuring changes in γ-aminobutyric acid levels in brain after administration of hydroxylamine (58).

Methods.—Reddy and co-workers (71) have modified the fluorometric method of Huff & Perlzweig (72) for the determination of 4-pyridoxic acid in urine. They employed ion exchange resins to reduce the high blanks and measured 4-pyridoxic acid in urine at concentrations as low as 0.5 μg./ml.

Levine & Hansen (73) have reported a new color reaction for pyridoxal, which is based upon its interaction with thiophene in the presence of strong sulfuric acid solution. An intense color was obtained with pyridoxal, but no color was obtained with pyridoxine, pyridoxamine, or pyridoxic acid.

Metabolism.—Rodwell *et al.* (74) and Ikawa *et al.* (75) have studied the metabolism of pyridoxal, pyridoxamine, and pyridoxine in various microorganisms, cultured to grow on one or more forms of the vitamin as the sole source of carbon and nitrogen. The metabolism of pyridoxine in one bacterial strain of the genus *Pseudomonas* was studied extensively. Four metabolites were isolated and identified as isopyridoxal, 5-pyridoxic acid, 5-pyridoxic acid lactone, and pyriconic acid. These findings point out a new route of vitamin-B_6 metabolism (Figure 3).

Reddy and co-workers (71) have confirmed the results of Rabinowitz &

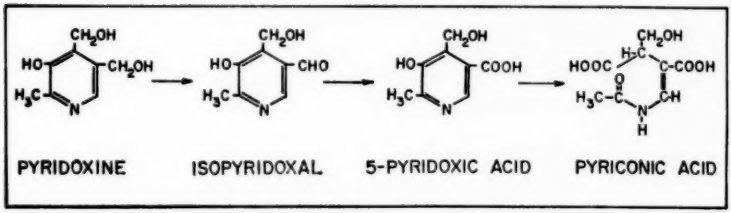

FIG. 3. Scheme for metabolism of vitamin B_6 postulated by Rodwell *et al.* (74) and Ikawa, Rodwell & Snell (75).

Snell (76), which show that 4-pyridoxic acid is the principal metabolite of pyridoxine in man. After the oral administration of 10 mg. of pyridoxine to human subjects, about 50 per cent of the dose was excreted in urine as 4-pyridoxic acid; no evidence was obtained for the excretion of conjugated 4-pyridoxic acid. Since a subcutaneous dose of 4-pyridoxic acid was recovered quantitatively in urine, further metabolism of this compound in man appears unlikely.

Sato *et al.* (77) presented evidence derived from both paper chromatographic and radioautographic studies indicating that both pyridoxine and pyridoxal were conjugated with S^{35}-sulfate in rat liver slices and homogenates. They suggest that the sulfate group is attached to the phenolic group of these compounds.

Miscellaneous.—Holden (78) reported that vitamin-B_6 deficiency markedly reduced the total amount of freely-extractable glutamic acid, which was accumulated by *Lactobacillus arabinosis* from buffers containing this amino acid and glucose. The effect of deficiency on the accumulation capacity was believed to be indirect and not attributable to the ineffective operation of a vitamin B_6-dependent catalyst functioning directly in the uptake process. Holden (79) has recently observed normal glutamate accumulation in vitamin B_6-deficient L-*arabinosus* when the osmotic pressure of the incubation media was increased. From these results, he suggested that the reduced glutamate accumulation in the deficient cells was caused by the instability of a cell structure.

Several studies have been carried out on the physicochemical properties of pyridoxal and its analogues. Heinert & Martell (80) investigated the nature of the hydrogen bond between the hydroxyl and the formyl groups of pyridoxal. In this study, the infrared spectra of pyridoxal and pyridoxal analogues were measured in the solid state and in carbon tetrachloride. King & Lucas (81) have determined the stability of some pyridoxal phosphate-amino acid complexes, expressed in terms analogous to the Michaelis constant. Bonavita & Scardi (82) have estimated spectrophotometrically the

various pK_a values of pyridoxal phosphate and its isonicotinylhydrazone and cyanohydrin derivatives.

Nicotinic Acid

Conversion of tryptophan to nicotinic acid.—The metabolism of tryptophan to nicotinic acid has recently been reviewed (83). The enzymatic steps required for conversion of tryptophan to nicotinic acid are known, except for the conversion of 3-hydroxyanthranilic acid to nicotinic acid. Moline *et al.* (84) reported that 3-hydroxyanthranilic acid-3-C^{14} was converted quantitatively by rat liver enzymes to quinolinic acid labeled in the alpha-carbon atom. Their results and those of Wiss & Bettendorf (85) suggest that 3-hydroxyanthranilic acid is converted to nicotinic acid through the intermediates shown in Figure 4. The formation of nicotinic acid from quinolinic

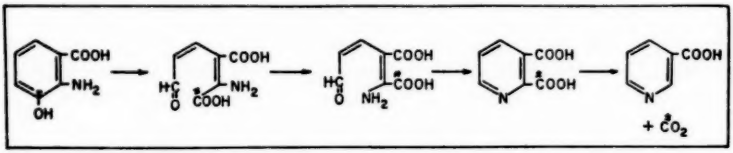

Fig. 4. Scheme for the conversion of 3-hydroxyanthranilic acid to nicotinic acid as postulated by Moline *et al.* (84).

acid, however, was not demonstrated. Decker & Henderson (86) have presented evidence from nutritional experiments for the conversion of 3-hydroxyanthranilic acid to nicotinic acid in the chick, guinea pig, and hamster.

Several studies have been carried out in man on the metabolism of tryptophan to nicotinic acid. Price (87) has administered loading doses of tryptophan to normal subjects and to patients with various clinical conditions and measured the urinary excretion of N-methyl-2-pyridone-5-carboxamide, kynurenine, kynurenic acid, acetylkynurenine, 3-hydroxykynurenine, xanthurenic acid, anthranilic acid glucuronide, and *o*-aminohippuric acid. He reported abnormal excretion patterns of these metabolites in pregnancy and in patients with porphyria, disseminated lupus erythematosus, scleroderma, vitamin-B_6 deficiency, and carcinoma of the urinary bladder. When increasing amounts of tryptophan were provided in a diet low in niacin, studies by Vivian *et al.* (88) with human subjects showed that the amino acid was used for niacin synthesis only after nitrogen balance was achieved. Brown *et al.* (89) fed human subjects a low niacin diet and measured the urinary excretion of 10 tryptophan metabolites. With increasing amounts of tryptophan in the diet, N-methyl-2-pyridone-5-carboxamide excretion remained at low levels until blood pyridine nucleotide levels were restored. The excretion of the other tryptophan metabolites more closely followed the tryptophan in-

take. Gassman & Knapp (90) have administered loading doses of tryptophan to normal and vitamin B_6-deficient subjects and measured the urinary excretion of kynurenine, 3-hydroxykynurenine, xanthurenic acid, N-methylnicotinamide, and N-methyl-2-pyridone-5-carboxamide. From the excretion patterns observed for these metabolites, they suggested the possibility of a new pathway of nicotinic acid synthesis in man.

Although tryptophan is a precursor of nicotinic acid in *Neurospora* and in mammals, the pathway for synthesis of the vitamin in plants is not known. Yanofsky's (91) results exclude a niacin-precursor role of tryptophan in bacteria. More recently, Henderson *et al.* (92) provided evidence that neither labeled tryptophan nor labeled 3-hydroxyanthranilic acid serve as a precursor of nicotinic acid in corn. These workers have also confirmed earlier studies indicating that tryptophan is not a precursor of nicotinic acid in tobacco.

Biosynthesis of diphosphopyridine nucleotide.—Evidence has accumulated recently indicating that nicotinic acid nucleotides are intermediates in the biosynthesis of DPN from nicotinic acid and nicotinamide. Preiss & Handler (93, 94) have studied the enzymatic reactions required for DPN synthesis in extracts of human erythrocytes, yeast autolysates, and preparations of rat liver. They concluded that nicotinic acid was converted to DPN as follows:

$$\text{Nicotinic acid} + \text{PRPP} \rightarrow \text{desamido-NMN} + \text{PP}_i$$

$$\text{ATP} + \text{desamido-NMN} \rightleftarrows \text{desamido-DPN} + \text{PP}_i$$

$$\text{Desamido-DPN} + \text{glutamine} + \text{ATP} \rightarrow \text{DPN} + \text{glutamate} + \text{AMP} + \text{PP}_i.$$

The enzyme that condenses nicotinic acid mononucleotide with ATP to form desamido-DPN was localized in the nuclear fraction of rat liver homogenate and appears similar to the DPN-pyrophosphorylase enzyme previously described by Kornberg (95). The enzyme responsible for the amidation of desamido-DPN to DPN was concentrated in the supernatant fraction of rat liver homogenate. Threlfall (96) found that rat and mouse liver slices metabolized nicotinamide, but not nicotinic acid, to DPN through the intermediate formation of desamido-DPN. Langan *et al.* (97) isolated desamido-DPN from the livers of mice injected with C^{14}-nicotinamide. Their studies furnish evidence that this nicotinic acid analogue is an intermediate in the synthesis of DPN from nicotinamide, nicotinic acid, and other pyridine compounds.

Kaplan *et al.* (98) reported that the amount of DPN in mouse liver increased markedly upon the injection of large doses of nicotinamide. Shuster & Goldin (99, 100) have found that administration of nicotinamide increased the incorporation of C^{14}-labeled glucose, formate, or glycine into free adenylic acid as well as into DPN. These findings suggest that the nicotinamide-induced synthesis of DPN resulted from a net synthesis of adenine and ribose rather than from the mobilization of these precursors into the liver for DPN synthesis. Shuster & Goldin (101) also found that the conversion of

glucose to ribose under these conditions occurred primarily through the transaldolase-transketolase pathway.

A review by Shuster *et al.* (102) indicated that it is possible to use induced *in vivo* synthesis of DPN to study biochemical mechanisms as well as the action of drugs in the animal. Burton *et al.* (103) reported that the administration of reserpine or promazine prior to the injection of nicotinamide resulted in elevated levels of DPN in liver for a prolonged period of time. Bosch & Harper (104) have shown that increases in hepatic pyridine nucleotides after an injection of nicotinamide were considerably less in hyperthyroid rats or mice than in normal animals, while no inhibition in the response to nicotinamide was observed in dinitrophenol-treated rats.

Langan *et al.* (97) found that the injection of azaserine markedly inhibited the stimulatory effect of nicotinamide on DPN synthesis. Studies by Narrod *et al.* (105) showed that administration of azaserine alone caused a marked reduction in the normal levels of DPN in mouse liver. Hartman *et al.* (106) suggested that azaserine may interfere with the synthesis of nucleotide precursors of DPN. Preiss & Handler (94) showed that azaserine was an effective inhibitor of DPN synthetase which catalyzes the amidation of desamido-DPN.

Nicotinic acid metabolism and miscellaneous.—Chang & Johnson (107) have identified for the first time N-methyl-4-pyridone-5-carboxamide as a major metabolite of nicotinic acid in rat urine. Chaykin & Bloch (108) reported that nicotinamide-N-oxide was formed from nicotinamide, *in vivo*, by rats. These workers also showed that hog liver homogenate reduced nicotinamide-N-oxide to nicotinamide. The possibility was suggested that nicotinamide-N-oxide may function as a biological oxidizing agent.

Hunt & co-workers (109, 110) have continued their studies on the hydroxylation of nicotinic acid by *Pseudomonas fluorescens*. The nicotinic acid hydroxylase system in homogenates of crushed cells was associated with a particulate fraction resembling the cell wall and protoplast membrane of the organism. The enzyme was purified 50-fold by deoxycholate extraction of the particulate fraction followed by ammonium sulphate fractionation. When the cell wall protoplast membrane was mildly disrupted, about 1 to 3 per cent of the nicotinic acid hydroxylase activity was obtained in a fraction having a sedimentation coefficient of $\bar{s}$ 40. The nicotinic acid hydroxylase activity of this fraction was associated with a lipoprotein complex.

Kimura (111) has purified nicotinamidase from *Mycobacterium avium* 250-fold. This preparation was inactive toward other amides, such as L-asparagine, L-glutamine, or DPN. Studies were also carried out by Kimura (112, 113) on the enzymatic transfer of the nicotinyl group of nicotinamide to hydroxylamine in *Mycobacterium avium*.

Leone & Bonaduce (114) have purified 100-fold the soluble nucleotide nucleosidase from bull seminal fluid. The ratio of TPNase to DPNase in these preparations was 0.70. Streptococcal DPNase has been highly purified and

the interaction of the enzyme with anti-DPNase antibody was studied by Pace & Pappenheimer (115).

Anderson *et al.* (116, 117) have prepared and studied the properties of nine pyridine-substituted DPN analogues. Those analogues, which were able to replace DPN as a coenzyme in various dehydrogenase systems, contained the following pyridine bases: nicotinyl hydroxamic acid, nicotinic acid hydrazide, pyridine-3-aldoxime, 3-benzoylpyridine, 3-isobutyrylpyridine, and thionicotinamide. The DPN analogues that failed to be reduced in the dehydrogenase systems tested possessed the following pyridine bases: 3-aminopyridine, 3-acetamidopyridine, and 3-pyridylacryloamide.

A method was devised by Stein *et al.* (118) for the assay of pyridine nucleotide transhydrogenase activity by using acetylpyridine analogues of DPN and TPN. This method, which involved the transfer of electrons from reduced TPN to the acetylpyridine analogue of DPN, gave transhydrogenase activities considerably higher than those obtained by other methods.

The treatment of animal diets with ethylene oxide results in severe destruction of niacin. Windmueller *et al.* (119) found that ethylene oxide reacted with nicotinamide in aqueous solution at 25° to yield, after acidification with HCl, N′-(2-hydroxyethyl)-nicotinamide chloride. This compound possessed no nicotinic acid activity for *Lactobacillus arabinosus* or for the chick. By a similar reaction, nicotinic acid was converted to the betaine of N′-(2-hydroxyethyl) nicotinic acid by ethylene oxide. The findings that the nicotinamide of DPN was not affected by ethylene oxide may explain the retention of nicotinic acid activity in some natural products that have been treated with ethylene oxide.

Dietrich *et al.* (120) reported that the level of DPN and the activity of certain critical DPN-dependent enzymes were lower in the mammary adenocarcinoma 755 of the C 57 mouse than in most host tissues studied. Furthermore, they observed that after 6-amino-nicotinamide administration, the ratio of enzymatically inactive 6-amino-DPN to total DPN was higher in the tumor than in host tissues. It was suggested that these observations furnish a possible explanation for the action of 6-amino-nicotinamide to destroy selectively the tumor and not host tissues. The experimental evidence that 6-amino-DPN is unable to function as an electron acceptor (121) has been supported by quantum-mechanical calculations of Pullman & Pullman (122), who showed that the lowest empty molecular electronic orbital is placed much higher in the 6-amino-nicotinamide analogue of DPN than in DPN itself.

Biotin

Numerous reports have appeared on a role for biotin in various carboxylation and decarboxylation reactions, but the specific mechanisms by which biotin functions have not been known. During the past year, Lynen & coworkers (123) have made important observations which throw light on the

mechanism of action of biotin. They found that the enzyme, β-methylcrotonyl-CoA carboxylase, contains biotin and that the vitamin is the active group of the enzyme. The carboxylase, which was prepared from cell-free extracts of *Mycobacterium* grown on isovaleric acid, catalyzed the following reaction:

$$CH_3{-}C(CH_3)=CH{-}CO{-}CoA + CO_2 + ATP \rightleftarrows HOOC{-}CH_2{-}C(CH_3)=CH{-}CO{-}CoA + ADP + P_i$$

Several lines of evidence indicated the presence of biotin in this carboxylase. The purified enzyme, when subjected to acid hydrolysis, was found to contain biotin when assayed by a yeast growth test. During purification of the enzyme, the biotin content and enzyme activity increased in the same ratio. It was also found that the potent biotin binder, avidin, inhibited the activity of the purified carboxylase and that this inhibition was overcome by the addition of biotin.

Exchange experiments with radioactive compounds by Lynen *et al.* (123) have furnished evidence for the formation of an "active" CO_2 intermediate during the carboxylation of β-methyl-crotonyl-CoA. The following sequence of reactions was proposed:

$$ATP + \text{biotin-enzyme} \overset{Mg^{++}}{\rightleftarrows} ADP \sim \text{biotin-enzyme} + P_i$$

$$ADP \sim \text{biotin-enzyme} + CO_2 \overset{Mg^{++}}{\rightleftarrows} CO_2 \sim \text{biotin-enzyme} + ADP$$

$$CO_2 \sim \text{biotin-enzyme} + \beta\text{-methyl-crotonyl-CoA} \rightleftarrows \text{biotin-enzyme} + \beta\text{-methyl-glutaconyl-CoA}$$

It was found that biotin could substitute for β-methyl-crotonyl-CoA as substrate for the carboxylase and that a biotin $\sim CO_2$ compound was formed. These investigators employed this compound as a model to study the "active" CO_2 intermediate in the enzymatic reaction, and it has one of the structures shown in Figure 5. The dimethyl ester of the enzymatically prepared biotin $\sim CO_2$ compound was shown by carrier dilution and paper chromatographic techniques to be identical to chemically synthesized N-

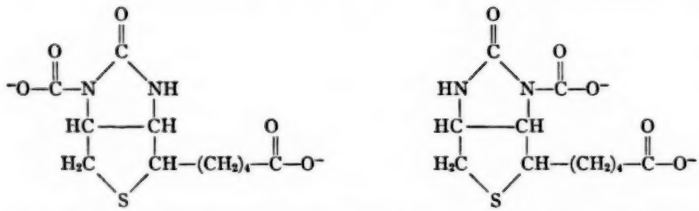

FIG. 5. Possible structure of the biotin $\sim CO_2$ compound given by Lynen *et al.* (123).

carbomethoxy-biotin-methyl-ester. Lynen *et al.* (123) have suggested that there may be many biotin-containing enzymes, just as there are many flavin-containing enzymes. Thus, biotin would transfer CO_2 in the different biotin-containing enzymes by its reversible uptake and output of CO_2.

Wakil & co-workers (124 to 126) have studied the enzymes in avian liver that catalyze the conversion of acetyl-CoA to palmitic acid, and have presented evidence for a malonic acid derivative as an intermediate in this reaction. The carboxylation of acetyl-CoA to the malonyl derivative was catalyzed by a biotin-containing enzyme in the presence of ATP, Mg^{++}, and CO_2. Biotin was shown to be present in this enzyme by microbiological assay and by inhibition experiments with avidin.

The results obtained by several groups indicated that biotin is not bound to certain enzymes involved in CO_2 transformation reactions. Tietz & Ochoa (127) have purified 500-fold a propionyl-CoA carboxylase from pig heart extracts and obtained no evidence for the presence of biotin in the purified carboxylase or for the occurrence of a separate enzymatic step for CO_2 activation. Hamilton & Westheimer (128) have isolated acetoacetic decarboxylase in crystalline form from *Clostridium acetobutylicum*. After hydrolysis of the decarboxylase, no bound biotin was detected. Ravel *et al.* (129) purified sixtyfold the ornithine-citrulline enzyme of *Streptococcus lactis* and observed that biotin was not a component of the enzyme. They suggested that the vitamin may exert its action by promoting the synthesis of the enzyme.

Lichstein (130) has studied the effect of biotin and several of its analogues on succinic acid decarboxylation and glucose fermentation by intact cells of *Propionibacterium pentosaceum*. Dethiobiotin was able to replace biotin for both glucose fermentation and succinate decarboxylation, whereas oxybiotin substituted for the vitamin in the former system and only to a limited extent in the latter system. Strauss & Moat (131) have studied the relationship of biotin deficiency to glucose oxidation in *Saccharomyces cerevisiae*. The results indicated that the vitamin stimulated hexokinase activity. Addition of biotin to the yeast extract increased CO_2 production from glucose or fructose but not from glucose-6-phosphate, fructose-6-phosphate, or hexose diphosphate.

Katsuki (132, 133) showed decreased metabolism of pyruvic acid and decreased levels of ATP and DPN in biotin-deficient *Piricularia oryzae* and *Bacillus macerans*. The addition of ATP or DPN restored α-keto acid oxidation in *B. macerans*. The accumulation of pyruvate in these deficient microorganisms was related to reduced oxidative phosphorylation. Friedman & Moat (134) have carried out studies with several microorganisms that need either biotin or preformed purines for growth. A requirement for the vitamin in the carboxylation of 5-amino-imidazole-ribotide to 5-amino-4-imidazole carboxylic acid ribotide was suggested. Biotin was also inferred to be involved indirectly in the subsequent metabolism of the carboxylated product to purines through its effect on aspartic acid synthesis. Bettrex-Galland (135) has studied the fixation of $C^{14}O_2$ into free amino acids of avian liver homo-

genates from normal and biotin-deficient animals. Aspartic acid was found to contain the major fraction of the radioactivity. Evidence was presented that the activities of two enzymes, oxalacetic decarboxylase and the malic enzyme, which are required for CO_2 fixation into aspartic acid, were reduced in biotin deficiency.

Schreier & Pfisterer (136) have studied the metabolism of biotin (carboxyl-C^{14}) in normal and biotin-deficient rats. The incorporation of C^{14} into respiratory CO_2, urine, and tissues was measured. The results indicated that the biotin-deficient rats retained somewhat larger quantities of the administered C^{14} than the normal animals.

Traub (137) has postulated that the biological activity of biotin may be related to its ability to form an intramolecular hydrogen bond between the keto oxygen of the ureido ring and one of the carboxyl oxygen atoms of the side chain. It was suggested that the formation of the hydrogen bond might alter the charge distribution in the ureido ring system and displace the keto-enol equilibrium to enol, resulting in a change of chemical reactivity at the nitrogen atoms. This concept is of interest because of the findings of Lynen *et al.* (123) indicating that biotin functions as a CO_2 carrier through a carboxylation reaction on one of these nitrogen atoms.

Inositol

Metabolism of inositol.—Charalampous (138) has continued his studies on the enzymes in rat kidney extracts that convert inositol to D,L-glucuronic acid. The enzyme that cleaves inositol to D-glucuronic acid but not to L-glucuronic acid was purified about 450-fold from rat liver extracts, and it was found to require sulfhydryl groups for activity and to contain iron. Burns *et al.* (139) have studied the metabolism of inositol-H^3 and inositol-C^{14} in rats. The results showed the formation of labeled D-glucuronic acid and L-gulonic acid in intact animals and in kidney extracts, but no labeled L-glucuronic acid was detected. Richardson & Axelrod (140) have also presented evidence for the metabolism of inositol through D-glucuronic acid and L-gulonic acid in rat kidney extracts, but they noted possible differences in inositol metabolism when whole kidney homogenates were used. Moscatelli & Larner (141) measured in rats the incorporation of photosynthetically prepared inositol-C^{14} into respiratory CO_2, organ lipids, and glycogen.

In view of the results showing that inositol is converted to D-glucuronic acid and L-gulonic acid, it now appears that the glucuronic acid pathway (Figure 1) may be a major route for inositol metabolism in animals. This is supported by the studies of Posternak *et al.* (142) and Anderson & Coots (143), in which the distribution of isotope in glucose from animals given specifically labeled inositol was measured. It should be noted that no conversion of labeled inositol to L-ascorbic acid was detected in rats; this observation was explained in terms of the organ distribution of enzymes involved in the catabolism of inositol and in the biosynthesis of L-ascorbic acid (139).

Anderson *et al.* (144) also reported that large doses of inositol had no antiscorbutic activity in guinea pigs.

Inositol phosphatides.—The discovery by Kennedy & Weiss (145) of the mechanism for the synthesis of phosphatidyl choline (lecithin) via CDP-choline and α,β,-diglyceride stimulated investigation of the formation of phosphatidyl inositol. Using a particulate preparation of guinea pig kidney, Agranoff *et al.* (146) noted a cytidine nucleotide requirement for the incorporation of inositol-H^3 into phosphatidyl inositol. Agranoff *et al.* (147) further suggest formation by kidney mitochondria of a "liponucleotide," tentatively identified as CDP-diglyceride. This compound was enzymatically cleaved by inositol to form phosphatidyl inositol and CMP. In experiments measuring the net formation of phosphatidyl inositol from L-α-glycerophosphate-P^{32} in liver microsomes, a specific requirement for CTP was observed by Paulus & Kennedy (148). These workers (149) have recently synthesized CDP-diglyceride, which undergoes enzymatic cleavage in the presence of inositol to form phosphatidyl inositol. According to Paulus & Kennedy, the overall formation of inositol monophosphatide occurred in the presence of an acylating system (ATP, CoA, and oleic acid) as follows:

$$\text{CTP} + \text{L-}\alpha\text{-glycerophosphate} + 2\text{RCO CoA} \rightarrow \text{CDP-diglyceride}$$

$$\text{CDP-diglyceride} + \text{inositol} \rightarrow \text{inositol monophosphatide} + \text{CMP}$$

Hokin & Hokin (150) observed in studies with guinea pig brain cortex slices that the incorporation of inositol-2-H^3 and of P^{32} into monophosphoinositide was increased in the presence of acetyl choline. Since both the labeled inositol and phosphate were incorporated to approximately the same extent, they suggested that acetyl choline stimulated the incorporation of labeled inositol monophosphate into monophosphoinositide. Incorporation of glycerol-C^{14} into monophosphoinositide, however, was not increased in the presence of acetyl choline.

The structure of phosphatidyl inositol from various sources has been investigated by Ballou & Pizer (151), Pizer & Ballou (152), and Brockerhoff & Hanahan (153), and it now appears that the point of attachment of the phosphate on inositol is to position one and not to position two, as was previously thought. Wagenknecht *et al.* (154) have described the isolation of the mixed calcium-magnesium salt of phosphatidyl inositol from lyophilized raw peas by a relatively simple solvent fractionation procedure. Elementary analyses and data on the hydrolysis products indicated that the parent compound was a monophosphoinositide, which probably possessed a phosphatidyl inositol structure. The fatty acids were shown to have C_{16} and C_{18} chain lengths. Hawthorne & Hübscher (155) reported the separation of glycerylphosphoryl inositol and related compounds on ion exchange columns. This work established the structure of the inositol-containing ester from liver phosphatides as glycerylphosphoryl inositol.

A requirement for phosphatidyl inositol or polyglycerol phospholipid for

the action of phospholipase B from *Penicillium notatum* on purified lecithin was observed by Dawson (156). In further studies, Bangham & Dawson (157) showed that phosphatidyl inositol and polyglycerol phospholipid presumably act by increasing the net negative charge of the lecithin particles so that they may be acted upon by the enzyme. Kemp *et al.* (158) isolated from rat liver a phospholipase which catalyzed the hydrolysis of monophosphoinositide to diglyceride and to inositol monophosphate. The action of this phospholipase was specific for those phospholipids that contained inositol.

Methods.—Kean & Charalampous (159) have developed two methods for the determination of inositol. Both methods rely on the measurement of the glucuronic acid formed from inositol by an inositol oxidase preparation from rat kidney extract. In one method, the resulting glucuronic acid was measured by the orcinol method; in the other, the enzymatic reduction of glucuronic acid by TPNH was followed spectrophotometrically.

A method for the determination of inositol was described by Agranoff *et al.* (147). It is based upon the oxidation of inositol with sodium periodate. The reduction of periodate is then measured spectrophotometrically.

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WATER-SOLUBLE VITAMINS, PART II[1]

(VITAMIN B_{12}, FOLIC ACID, THIAMINE, RIBOFLAVIN, PANTOTHENIC ACID)

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Because of space limitations, no effort has been made to review all the literature which has appeared over the past year. Papers have been selected which deal primarily with biological applications of the water-soluble vitamins.

VITAMIN B_{12}

Determination of vitamin B_{12}.—Several modifications of the microbiologic determination of vitamin B_{12} have been reported, and several precautions which must be taken in evaluating the results have been pointed out. Cooper (1) has reported a B_{12} assay procedure utilizing *Euglena gracilis* which shortens the assay time considerably. Additional improvements in the microbiologic assay of vitamin B_{12} with *E. gracilis* have been reported by Nicholas & Pitney (2). A report by Fries (3) suggests the possibility of use of the marine red algae *Goniotrichum elegans* as an assay organism for vitamin B_{12}.

The loss of vitamin B_{12} during autoclaving has been studied by Mukherjee & Sen (4, 5). They have pointed out that, during autoclaving at pH 8, there is considerable destruction of vitamin B_{12} and that, during autoclaving at pH 4 to 4.5 in the presence of thiamine and nicotinamide, B_{12} is also lost. The inclusion of ferric chloride in the solution during autoclaving appeared to offer protection for vitamin B_{12}.

Spray & Taylor (6) studied the assay of rat-serum B_{12} with *Lactobacillus leichmannii*. It was found that, whereas alkali treatment resulted in destruction of crystalline B_{12}, there was considerable growth-stimulating activity for *L. leichmannii* after alkali treatment of rat serum. They noted that, to determine true B_{12} of rat serum, it is necessary to assay with the organism before and after alkali treatment.

Biosynthesis of vitamin B_{12}.—Bray & Shemin (7) have studied the biosynthesis of the B_{12} molecule by microorganisms. It was found that δ-aminolevulinic acid was utilized for synthesis of the porphyrin moiety. However, vitamin B_{12} has additional methyl groups which cannot arise from the δ-aminolevulinic acid. It was found that, in a strain of *Actinomycetes*, the methyl group of C^{14} methyl-labeled methionine was transferred to a significant degree to B_{12}, thereby suggesting a direct methylation. C^{14}-labeled

[1] The survey of literature pertaining to this review was concluded October 1, 1959.

porphobilinogen was incorporated into B_{12} by bacteria in studies by Schwartz *et al.* (8). They suggested that the biosynthesis of the porphyrin moiety of B_{12} probably proceeds along the same pathways as other porphyrin syntheses.

There have been several reports on the content of vitamin B_{12} in various biological materials. Braekkan (9) determined the B_{12} concentration of organs from various marine fish, and Rao *et al.* (10) the B_{12} content of various sewage sludges. It was found by Andrews *et al.* (11) that livers of lambs receiving rations low in cobalt and exhibiting cobalt-deficiency signs were quite low in B_{12} content. Gray & Daniel (12) made the interesting observation that turnip greens from a certain location contained B_{12} activity for microorganisms; this was in contrast to the findings in other higher plants. Electrophoretic and chromatographic data indicated that this substance was actually vitamin B_{12}. The origin of this B_{12} is not known, although it would appear likely that it arises from bacterial action.

Intrinsic factor, B_{12} absorption, and B_{12} binding by tissues.—In spite of a large amount of work over the past year, the exact mechanism of intrinsic factor action in B_{12} absorption is far from clarified. To date, there appear to be no pure intrinsic factor preparations available. Schwartz *et al.* (13) have again pointed out a possible species specificity of intrinsic factor preparations. It was found that prolonged treatment of pernicious anemia patients with preparations of hog pyloric mucosa and oral vitamin B_{12} led eventually to failure of absorption of the B_{12}. Intrinsic factor from another species (rat) was effective.

Possible site of production of intrinsic factor in the rat has been studied by Keuning *et al.* (14). After resecting various regions of the rat stomach and studying B_{12} absorption, they concluded that the fundus was the site of intrinsic factor production.

There have been continued efforts to devise an *in vitro* system in which B_{12} binding can be related to clinical intrinsic factor activity. It appears that this aim has not yet been achieved. Berlin *et al.* (15) reported that, with partially purified intrinsic factor, the intrinsic factor activity as measured by the Schilling test agreed quite well with B_{12}-binding activity as measured with *Escherichia coli*. Barlow & Frederick (16) subjected intrinsic factor preparations to paper electrophoresis in the presence of radioactive B_{12} and found that one of the binding sites agreed quite well with clinical intrinsic factor activity. Rhodes, Feeney & Miller (17) fractionated intrinsic factor preparations on a cellulose column. The various fractions were assayed by an *in vitro* procedure (combination of B_{12} with a receptor protein) and for clinical activity by the Schilling test. Slight differences were found in the peaks of activity by the two assay procedures. Gräsbeck (18) treated intrinsic factor with various chemicals and enzymes and measured the effect on B_{12} binding and on intrinsic factor activity. Every treatment that inhibited B_{12} binding inhibited intrinsic factor activity. Acetylation did not inhibit binding but did inhibit intrinsic factor activity. It was concluded that binding of B_{12} was

prerequisite for intrinsic factor activity and that, in addition, another part of the intrinsic factor molecule was necessary for physiological activity.

It must be pointed out, however, that many tissues possess the capacity to bind vitamin B_{12} and do not demonstrate intrinsic factor activity. Meyer *et al.* (19) found that B_{12} was bound by cerebral spinal fluid; Gräsbeck & Takki-Luukkainen (20) found that tear fluid would bind vitamin B_{12}; and Bertcher, Meyer & Miller (21) reported additional evidence for the binding of B_{12} by saliva. It appears that, although significant progress has been made, there is still no satisfactory *in vitro* procedure for assaying clinical intrinsic factor activity.

There have been several reports on the effects of sorbitol on B_{12} absorption, and the overall picture is not clear. Chow *et al.* (22) have continued their studies and present additional data indicating that sorbitol will enhance B_{12} absorption in all but pernicious anemia patients. Boger *et al.* (23) support this finding of enhanced B_{12} absorption by sorbitol. They also found that the administration of sorbitol without B_{12} did not influence serum-B_{12} levels. This is interpreted to mean that sorbitol does not exert its effect by an influence on the flora of the gastrointestinal tract. Herbert *et al.* (24) and Boger & Parmelee (25) likewise found a slight effect of sorbitol on the absorption of vitamin B_{12}, while Chalmers & Shinton (26) found no such effects with either normal or aged subjects. Ellenbogen, Herbert & Williams (27) reported that sorbitol did not influence the absorption of B_{12} by pernicious anemia patients either in the presence or absence of intrinsic factor. From all available reports, it appears that sorbitol does not influence the absorption of B_{12} by patients with pernicious anemia and has only a slight effect on the absorption of B_{12} by normal subjects.

There have been conflicting reports of the effects of ethylenediaminetetraacetate on B_{12} absorption. It has been reported to inhibit the absorption of B_{12} in man (28) and to have no influence on the absorption of B_{12} in gastrectimized rats (29). It is apparent that additional work will be necessary to clarify the effect of this calcium-chelating agent on B_{12} absorption.

The anatomical site of absorption of B_{12} from the gastrointestinal tract has been studied by several groups, and the available evidence is consistent with the idea that the chief site of absorption of B_{12} in both man and dogs is the ileum (30, 31, 32). It has been suggested that B_{12} is temporarily stored in the intestinal wall during absorption (33).

The protein responsible for the binding of B_{12} by rat serum has been studied by Mulgaonkar & Sreenivasan (34, 35). They have found that, when physiological doses of B_{12} are added to rat serum, the binding is almost exclusively in the alpha-2-globulin, but that when large quantities of B_{12} are added, the B_{12} is bound by other proteins, notably gamma-globulin. It appears that the physiological binding of B_{12} is to the alpha-2-globulin; however, when large doses are added, other and perhaps all plasma proteins may exhibit binding activity.

There has been additional evidence that, in liver disease, the binding of

B_{12} is impaired. Glass *et al.* (36) reported that a large number of patients with liver disease exhibited an impaired uptake of Co^{60}-labeled B_{12} by the liver, while Stevenson & Beard (37) found that patients with liver disease exhibited elevated serum-B_{12} levels but no change in B_{12} absorption. They postulate that, in liver disease, the vitamin is released from the liver because of defective binding.

Several reports indicate that the uptake of vitamin B_{12} by various tissue preparations, *in vitro*, is enhanced by the addition of intrinsic-factor. Driscoll *et al.* (38) obtained fractions of human gastric contents from an ion-exchange column which would enhance the B_{12} uptake by rat liver slices. Similar results were obtained by Latner & Raine (39), who found the uptake of B_{12} by rat liver slices was enhanced by the addition of intrinsic factor. Strauss & Wilson (40) and Herbert (41) found that the uptake of B_{12} by everted sacs of rat small intestines was stimulated by intrinsic factor.

There have been some suggestions of a relationship of vitamin B_{12} to leukemia. Doctor *et al.* (42) found a fair correlation between serum-B_{12} levels and leucocyte counts in leukemia patients undergoing therapy. Miller & Sullivan (43) studied B_{12} binding by sera from normal patients and patients with leukemia and found certain differences in the physiochemical properties of the binding protein. Rosenthal (44) suggested that B_{12} is incorporated into the red cells during their maturation in the bone marrow and is not incorporated into mature erythrocytes. A note of caution on the use of Co^{60}-labeled B_{12} is made by Smith (45), who found that the radioactive B_{12} is quite unstable and should be checked for radiochemical purity at least monthly.

The metabolic role of vitamin B_{12}.—There is no satisfactory, readily available antagonist of vitamin B_{12} to be used in metabolic studies. Baker *et al.* (46) reported the preparation of several substituted amides of B_{12} and their action in microorganisms. They were considerably less active than B_{12} for growth of the microorganisms, but there was no evidence that they acted as an antagonist. Funk & Nathan (47) found that benzimidazole would inhibit the growth of two B_{12}-requiring algae. However, this inhibition was not competitive, and the data do not prove that the growth inhibition was caused by interference with the utilization of vitamin B_{12}.

Weissbach, Toohey & Barker (48) have continued their studies on the B_{12} coenzyme isolated from *Clostridium tetanomorphum*. This coenzyme is required for the conversion of glutamate to β-methylaspartate. They found three forms of active coenzyme: benzimidazole B_{12}, adenine B_{12}, and dimethylbenzimidazole B_{12}. The latter was isolated from rabbit liver and is believed to constitute a major portion of the total B_{12} content of that tissue.

There is some evidence indicating a role of B_{12} in lipid metabolism, although the exact mechanism is not clear. Spivey-Fox *et al.* (49) described a purified diet useful in producing B_{12}-deficiency in rats. Increasing levels of dietary fat precipitate B_{12} deficiency. Yesair *et al.* (50) determined the phospholipid content of developing chick embryos as related to B_{12} nutrition but they found no striking effect of B_{12} on phospholipid content. Newberne & O'Dell (51, 52) studied the influence of maternal B_{12} intake on various patho-

logic and physiologic changes of the offspring. The offspring from B_{12}-deficient rats exhibited macrocytic anemia and leucopenia. Pathologic changes in the central nervous system were also observed. Under their conditions, there is evidence of deficiency in myelination in the spinal cord but no defect in myelination in the brain. Since one of the striking signs of B_{12}-deficiency in man is demyelination, it is important that some experimental model be available to study the influence of B_{12} on myelin formation.

The possible need for B_{12} for adequate storage of folic acid is indicated by Cox *et al.* (53), who found that the excretion of folic acid in the urine following a test dose was lower in pernicious anemia patients than normal patients, and by Dawbarn *et al.* (54), who found that sheep deficient in B_{12} exhibited low liver levels of folic acid and citrovorum factor which could not be related to reduced food intake.

There was a suggestion several years ago that vitamin B_{12} may be involved in the metabolism of sulfhydryl compounds. There has been a minimum of data to support this hypothesis, and any such effect of vitamin B_{12} remains to be unequivocally demonstrated. Kato & Murakami (55) reported that carbon tetrachloride administration to rabbits results in increases in serum B_{12} and decreases in liver B_{12}. Similar results were obtained by Kasbekar *et al.* (56), who found, in addition, that the low levels of liver B_{12} were accompanied by depletion in liver sulfhydryl compounds. These workers suggested that B_{12} protects the carbon tetrachloride-treated rats by virtue of an effect on sulfhydryl compounds. Kasbekar *et al.* (57) came to a similar conclusion with respect to the effect of thyroid feeding. Thyroid-fed rats exhibited low levels of liver and blood glutathione, and dietary administration of B_{12} raised these levels. They suggested that B_{12} protected the rats by maintaining mitochondrial integrity through an effect on sulfhydryl compounds. Hsu, Chow & Okuda (58) studied the effects of B_{12} supplementation on glutathione content of erythrocytes and liver in chicks. B_{12} administration exerted no convincing effect.

The possible role of vitamin B_{12} in protein biosynthesis has continued to be investigated by Wagle *et al.* (59) and Mehta *et al.* (60). They found that the addition, *in vitro*, of Barker's coenzyme (48) stimulated the incorporation of C^{14} phenylalanine into proteins by a combination of microsomes and pH-5 enzymes from B_{12}-deficient rats. The coenzyme was considerably more active than crystalline B_{12}. They further reported, in this brief note, in contrast to earlier reports from their laboratory, that their current data indicate that B_{12} is not involved in the activation of amino acids. These observations of an influence of B_{12} on protein biosynthesis were not supported by the English workers. Fraser & Holdsworth (61) and Arnstein & Simken (62) were unable to find any significant effect of vitamin-B_{12} deficiency on the incorporation of amino acids into proteins by chicks or rats, nor was there any striking *in vitro* effect of B_{12} on this reaction. Dinning & Young (63) likewise were unable to obtain any evidence for enhancement by B_{12} of the incorporation of amino acids into protein by B_{12}-deficient chick bone-marrow preparations.

Studies on the role of B_{12} in thymine biosynthesis have been continued by

Dinning & Young (63). Bone-marrow cells from chicks deficient in vitamin B_{12}, when incubated with deoxyuridine and sodium formate C^{14}, converted formate to thymine methyl at a considerably reduced rate as compared with chicks receiving weekly injections of vitamin B_{12}. The addition of crystalline B_{12} to the B_{12}-deficient marrow cells restored to normal the rate of conversion of formate to thymine methyl. From these and other results from this laboratory, it has been concluded that vitamin B_{12} is concerned in the reduction of formate to thymine methyl and that the site of action of B_{12} is between the formate and formaldehyde levels of oxidation. Studies with purified enzymes will be necessary to elucidate the exact role of vitamin B_{12} in this transformation of one-carbon compounds.

It has been known for some time that in microorganisms vitamin B_{12} is involved in deoxyribose formation. It was observed many years ago that deoxyribosides would replace the B_{12} requirement for *Lactobacillus leichmannii*. In investigating this phenomenon, Spell & Dinning (64) studied the effect of B_{12} on the conversion of ribose to deoxyribose by *L. leichmannii*. When this organism was grown in the presence of B_{12}, deoxycytidine, and ribose-1-C^{14}, the ribose was converted to DNA deoxyribose at a rate which indicated that this was the sole source of DNA deoxyribose. In contrast, in cells grown without B_{12} (growth supported by deoxycytidine), there was no conversion of ribose to deoxyribose. From these results, it is concluded that B_{12} is required for the conversion of ribose to deoxyribose by *L. leichmannii*.

Folic Acid

Much of the progress made in the folic acid area during the past year has been in more complete elucidation of the various metabolic transformations of the folic acid group of compounds and of the chemical nature of the coenzyme forms.

Biosynthesis and requirements.—Shiota (65) incubated 2-amino-4-hydroxy-6-hydroxymethyl-tetrahydropterine, *p*-aminobenzoylglutamic acid, ATP, and magnesium with extracts of *Lactobacillus arabinosus*. Under these conditions, a substance was synthesized which exhibited folic acid activity for *Streptococcus faecalis*. Trager (66) studied the folic acid requirement for the nutrition of malaria parasites. The avian malaria parasite *Plasmodium lophurae*, when grown in tissue culture, would respond to folinic acid but not to folic acid. Apparently this organism cannot complete the transformation of folic acid to the reduced form. Campbell & Sniff (67) studied the folic acid requirement for several strains of *Bacillus coagulans*. It was found that the folic acid requirement for these organisms could be replaced by a variety of substances related to folic acid and, in some strains, the folic acid requirement could be replaced by vitamin B_{12}. Several strains were able to utilize xanthine or thymine for growth in lieu of folic acid. Sakami & Knowles (68) described a method for the purification of folic acid which may prove useful for its preparation for microbiologic and enzyme studies.

Metabolic transformations of folic acid compounds.—The existence of a

variety of substances with folic acid activity in blood has been reported by Usdin (69). Extracts of human red blood cells were chromatographed and the fractions assayed microbiologically for folic acid activity; at least nine peaks were reported. Somewhat similar findings were described by Toennies & Phillips (70) who reported additional experiments on the folic acid precursors found in red blood cells and on the folic acid activators or enzymes found in plasma. Fractionation studies indicated that there were several different substances in red blood cells which would serve as folic acid precursors and that the so-called plasma enzyme was of a multiple nature.

Wood & Hitching (71) studied the uptake of folic acid and citrovorum factor by several bacterial species. Glucose was essential for the uptake of both compounds and they suggested that this uptake was enzymatic in nature. It was also found that several of the bacterial species studied would degrade folic acid and citrovorum factor to a diazotizable amine, presumably *p*-aminobenzoylglutamic acid. Formation of a diazotizable amine from folic acid was also studied by Dawbarn, Hine & Smith (72), who found that, during microbiologic assay, there was cleavage of the citrovorum factor which prevented quantitative recoveries.

The conversion of folic acid to citrovorum factor has been studied by a number of workers. Doctor (73) studied the conversion of folic acid to citrovorum factor by rats in experiments, *in vivo*. Administration of vitamin C to the animals or the addition of vitamin C to the urine collection jars enhanced the yield of citrovorum factor to about the same extent. Evidence is accumulating that ascorbic acid does not actually stimulate the transformation of folic acid to citrovorum factor but merely protects the citrovorum factor from destruction. Estradiol stimulates the conversion of folic acid to citrovorum factor in male rats and this observation may be related to the recently accumulated evidence of a possible role of estradiol in TPN metabolism. Heisler & Schweigert (74) studied the conversion of folic acid to citrovorum factor by extracts of *Lactobacillus casei*. The reaction was stimulated by ascorbic acid, serine, and TPN and by an unknown factor present in boiled extracts of *L. casei*. They suggested that the co-factor was the polyglutamate form of citrovorum factor which was acting catalytically in the conversion of folic acid to citrovorum factor. Additional evidence on this point will be of considerable interest. There have been further reports showing that aminopterin inhibits the conversion of folic acid to citrovorum factor by liver (75) and by developing chick embryos (76).

The reduction of dihydrofolic acid to the tetrahydro form has been studied by Osborn & Huennekens (77). From acetone powder extracts of chick liver, an enzyme was partially purified which would catalyze the reduction of dihydrofolic acid. The enzyme required triphosphopyridine nucleotide (reduced form) (TPNH) and the over-all reaction was: 7,8-dihydrofolic acid + TPNH → 5,6,7,8-tetrahydrofolic acid + TPN. It had been shown earlier by Futterman (78) that the reduction of folic acid to tetrahydrofolic acid was mediated through a TPNH-dependent chick liver enzyme. Thus, it ap-

pears that the reduction of folic acid to the tetrahydro form is a two-step process and TPNH is the electron donor for each of these steps. Peters & Greenberg (79) also studied the enzyme dihydrofolic acid reductase and reported that either diphosphopyridine nucleotide (reduced form) (DPNH) or TPNH would serve in the reduction. DPNH was more effective at lower pH range while TPNH was more effective at pH values above six. Thus, the reports are, in general, in agreement and indicate that, under physiological conditions, TPNH is the most effective agent in this reduction. Peters & Greenberg (80) further demonstrated that aminopterin non-competitively inhibited the reduction of folic acid and diglutamyl folic acid by sheep liver.

In a very important paper, Hartman & Buchanan (81) reported on the chemical nature of the formyl donor in the biosynthesis of the purine ring. Folic acid compounds are known to function in the insertion of the one-carbon fragment into the 2 and 8 positions of the purine ring. It was found that 5-N, 10-N-anhydroformyltetrahydrofolic acid was a specific formyl donor for the carbon 8 of the purine ring, whereas 10-N-formyltetrahydrofolic acid was a specific formyl donor for the carbon 2 of the purine ring.

Blakley (82) studied the reaction of formaldehyde with tetrahydrofolic acid. It was found that a non-enzymatic reaction between tetrahydrofolic acid and formaldehyde resulted in the formation of 5-N, 10-N-methylene-tetrahydrofolic acid.

Tabor & Wyngarden (83) have elucidated the steps involved in the metabolism of formiminoglutamic acid to formyltetrahydrofolic acid. They partially purified from rabbit liver three enzymes which catalyzed the following steps:

$$\text{Formimino-L-glutamic acid} + \text{tetrahydrofolic acid} \xrightleftharpoons{\text{formimino-transferase}} \text{glutamic acid} + \text{5-formimino-tetrahydrofolic acid}$$

$$\text{5-Formimino-tetrahydrofolic acid} \xrightleftharpoons{\text{formimino-tetrahydrofolic acid cyclodeaminase}} \text{5,10-methenyl-tetrahydrofolic acid} + NH_3$$

$$\text{5,10-Methenyl-tetrahydrofolic acid} + H_2O \xrightleftharpoons{\text{cyclohydrolase}} \text{10 formyltetrahydrofolic acid}$$

Metabolic effects of folic acid compounds.—There have been reported a number of somewhat diverse effects of folic acid deficiency on metabolic reactions. Most of these probably are indirect consequences of the major function of folic acid in the transfer of one-carbon fragments.

Rohatgi & Banerjee (84) reported that aminopterin treatment in rats resulted in a reduction in plasma albumen and gamma-globulin and in an elevation in the alpha-2-globulin. Johnson, Corte & Jasmin (85) found that the addition of amethopterin (4-aminomethylfolic acid) or aminopterin strongly inhibited the acetylation of sulfanilamide by pigeon liver extracts. This inhibition was non-competitive. Folic acid in comparable concentration

was non-inhibitory. It was also found that, when these folic acid anti-metabolites were injected into animals, *in vivo* acetylation of sulfanilamide was inhibited.

Sauberlich (86) studied the effects of dietary B_{12} and folic acid on various aspects of methyl synthesis and transfer in mice. It was found that, in the mouse, dietary folic acid stimulated liver choline oxidase activity to a considerable extent. Faulkner, Blood & Darby (87) found that folic acid deficiency in the guinea pig did not lead to reduced myoglobin concentrations, although there was a significant reduction in hemoglobin.

The anti-malaria drug, pyrimethamine, was found to inhibit the conversion of folic acid to citrovorum factor by extracts of *Streptococcus faecalis* by Wood & Hitchings (88). This inhibition was non-competitive. Pyrimethamine was also found to interfere with folic acid metabolism in chicks. Lucas (89) reported that, when this substance was fed to chicks, they developed macrocytic anemia. This could be prevented by extra dietary folic acid.

Humphreys & Greenberg (90) studied the formation of thymine by thymus gland preparations. Deoxyuridine-5′-phosphate was converted to thymidine-5′-phosphate in the presence of C^{14} formaldehyde, and tetrahydrofolic acid served as a reducing agent in this reaction, being oxidized to dihydrofolic acid. The dihydrofolic acid was in turn reduced to tetrahydrofolic acid in the presence of DPNH.

The influence of folic acid deficiency on the excretion of formiminoglutamic acid has been studied by several investigators. Broquist & Luhby (91) reported that children with leukemia receiving folic acid antagonists and a patient with macrocytic anemia of pregnancy excreted formiminoglutamic acid. They suggested the use of formiminoglutamic acid excretion as a measure of folic acid deficiency. Silverman & Pitney (92) found that rats fed a low protein diet excreted large quantities of formiminoglutamic acid unless folic acid and B_{12} were given. The administration of methionine in the absence of folic acid and B_{12} also reduced formiminoglutamic acid excretion. Homocysteine was somewhat less effective than methionine. This influence of methionine on the excretion of formiminoglutamic acid is difficult to explain on the basis of present information. Luhby, Cooperman & Teller (93) studied the excretion of formiminoglutamic acid by patients with megaloblastic anemias caused by folic acid deficiency and by patients with pernicious anemia. Patients with megaloblastic anemias related to folic acid deficiency excreted large quantities of formiminoglutamic acid following a histidine-loading test. By contrast, pernicious anemia patients under the same conditions did not excrete measurable quantities of formiminoglutamic acid. It is suggested that this be used as a differential diagnosis for folic acid and vitamin-B_{12} deficiency in man. The results of Silverman & Pitney (92), indicate that B_{12} administration reduces formiminoglutamic acid excretion in rats. This is difficult to reconcile with the observation of Luhby *et al.* (93) which indicates a specific effect of folic acid deficiency on formiminoglutamic acid excretion.

Kaufman (94) studied the livers of patients with phenylketonuria to determine if there was a deficiency of the co-factor related to folic acid which is concerned in the enzymatic conversion of phenylalanine to tyrosine. Evidence obtained indicated that this co-factor was present in normal concentration in livers of patients with this disease.

Riboflavin

Maley & Plaut (95, 96) have continued their studies of riboflavin biosynthesis by *Ashbya gossypii*. It was found that an intermediate accumulated during the incorporation of formate C^{14} into riboflavin by this organism. This intermediate was isolated and identified as 6,7-dimethyl-8-ribityllumazine. Time studies on the incorporation of formate C^{14} into this substance indicated that it was an intermediate in the pathway of riboflavin biosynthesis rather than a degradative product. More direct evidence for this hypothesis was obtained when it was found that the incubation of 6,7-dimethyl-8-ribityllumazine with cell-free extracts of *A. gossypii* led to the net synthesis of riboflavin. The authors pointed out that the possibility exists that this substance may be a precursor of other pteridines, such as folic acid, as well as riboflavin. Katagiri, Takeda & Imai (97, 98) likewise studied the biosynthesis of riboflavin and specifically studied the biosynthesis of the intermediate 8-N-ribityl-6,7-dimethyllumazine. Goodwin & McEvoy (99) studied riboflavin biosynthesis in *Candida florieri*. Iron at high concentration inhibited riboflavin production; certain amino acids stimulated growth but inhibited riboflavin biosynthesis. There appeared to be an inverse relationship between growth of the organism and the quantity of riboflavin synthesized.

Miles *et al.* (100) studied the degradation of riboflavin by a riboflavin-decomposing aerobe. They isolated some intermediate products in the degradative pathway. Gershoff *et al.* (101) described riboflavin deficiency in the cat. They found that a high carbohydrate, low fat diet appeared to exert a sparing effect on the riboflavin requirement. This may be related to alterations in intestinal flora. Kalter (102) reported that riboflavin-deficient mice produced young with a variety of congenital malformations. This is most likely a non-specific effect, since a variety of insults during fetal development will lead to fetal malformations.

Two reports have appeared indicating an influence of riboflavin deficiency on tryptophan metabolism. Stevens & Henderson (103) reported that riboflavin deficiency in rats led to a reduced liver L-kynurine hydroxylase activity. *In vitro* additions of flavin coenzymes were without effect. Sylianco & Berg (104) studied the effects of riboflavin deficiency on the metabolism of tryptophan by rat liver and kidney slices. Riboflavin deficiency did not affect the conversion of indolepyruvic acid to tryptophan. However, the degradative pathways of tryptophan were drastically altered in the riboflavin-deficient slices. Among other changes, there was an increased production of xanthurenic acid; this is in agreement with certain *in vivo* observations which have been made in the past. There was also an increased accumu-

lation of kynurenic acid and anthranilic acid from tryptophan when incubated with liver slices from riboflavin-deficient animals.

Isenberg & Szent-Györgyi (105) studied the reaction between tryptophan and riboflavin. It was found that free tryptophan or tryptophan in proteins formed a complex with riboflavin in which the riboflavin molecule takes up one electron from the tryptophan and is then present as a free radical in the semiquinoid form. It is speculated that such a riboflavin-protein-tryptophan complex occurs *in vivo* and is significant in electron-transferring systems. Burn and O'Brien (106) studied the reduction of riboflavin by dithionate; an intermediate was formed during the reduction. Thus, it appears that a certain amount of progress has been made in studying riboflavin in model systems, a technique which has been used so successfully with pyridoxine and thiamine.

An interesting report appeared by Pirie (107). It was found that the tapetum of the lemur *Galago crassicaudatus* is composed of crystals of riboflavin. These lie behind the retina and serve to reflect unabsorbed light back to sensitive cells. Scala & Lambooy (108) found that, after sub-culturing *Lactobacillus casei* in media with minimum amounts of riboflavin and increasing amounts of 6-chloro-7-methyl-9 (*l*-D-ribityl) isoalloxazine, the organism would eventually accept the latter compound as its flavin source.

Thiamine

Baker *et al.* (109) described a microbiologic assay of thiamine by use of the protozooan *Ochromonas malhamensis*. A gravimetric silicotungstate method for the determination of thiamine has been described by Vannatta & Harris (110, 111). Interfering substances in biological materials were removed by ion exchange chromatography.

The biosynthesis of thiamine by yeast has been studied by Nose, Ueda & Kawasaki (112). When the phosphate esters of 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5-hydroxymethylthiazole were incubated with the partially purified enzyme from baker's yeast, thiamine was formed. They speculate that the monophosphate or diphosphate form of thiamine is produced in this reaction. Wright (113) reported that the microorganism *Mycobacterium butyricum* could synthesize the thiazole fragment of thiamine but not the pyrimidine fragment.

The degradation of thiamine by a microorganism was studied by Hosoda *et al.* (114). Additional evidence has appeared indicating the important role of intestinal microorganisms in thiamine metabolism in animals. Wostmann *et al.* (115) reported that the addition of penicillin to a thiamine-deficient diet for conventional rats improved growth and elevated liver thiamine stores. In contrast, the antibiotic did not influence growth or liver stores of thiamine in germ-free rats. These results indicate that the penicillin effect is related to alterations in the host microflora and are in agreement with the general consensus of opinion on the mode of action of antibiotics in promoting growth of animals. Jones & Baumann (116) reported that dietary sorbitol or

penicillin would stimulate the growth of rats receiving a purified diet devoid of thiamine. Again these results most likely reflect an effect on intestinal microorganisms.

Nath & Belkhode (117) studied the biosynthesis of ascorbic acid in germinating mung beans and found that thiamine and pantothenic acid would accelerate the biosynthesis.

Baldridge (118) reported that thiamine deficiency in rats led to an elevated liver histidase activity. It was suggested that this is an adaptive response to a defect in another pathway of histidine utilization that is dependent on thiamine. Wertman & Groh (119) reported that thiamine-deficient rats were markedly susceptible to *Diplococcus pneumoniae* infection as compared with animals receiving adequate thiamine. This susceptibility was not caused by a shortage in granulocytes or by reduction in phagocytic activity.

Wolfe, Brin & Davidson (120) reported that erythrocytes of thiamine-deficient patients exhibiting Wernicke's encephalopathy were unable to metabolize C^{14} glucose normally. The pattern of recovery of carbon dioxide from carbons 1 and 2 of the glucose indicated that these cells were deficient in transketolase, a thiamine-requiring step which is necessary for the pentose-phosphate pathway of glucose utilization.

There have been several reports of the reaction of thiamine in model systems and considerable information has accumulated regarding the exact mechanism of thiamine action in metabolic systems. Yount & Metzler (122) with Yatco-Manzo & Roddy (121) have continued their study of the mechanism of action of thiamine in model systems. They found that thiamine non-enzymatically catalyzed the decarboxylation of pyruvate to acetoin and α-acetolactate, and they studied the structural requirements for pyruvate decarboxylation in model systems. The thiazolium ring was necessary, whereas the pyrimidine ring was found to be unnecessary in this system.

Krampitz *et al.* (123) presented evidence for an active acetaldehyde-thiamine intermediate. DeTar & Westheimer (124) studied the mechanism of action of thiamine pyrophosphate in the enzymatic decarboxylation of pyruvate. The results with the enzyme system supported the proposed mechanism of action based on early experiments in model systems (125).

Pantothenic Acid

The herbicide 2,3-dichloroisobutyrate was found to inhibit the synthesis of pantothenic acid by an extract of *Escherichia coli* (126). Moffatt & Khorana (127) reported the total chemical synthesis of coenzyme A. In two important papers, Brown (128, 129) presents evidence for the pathway of the biosynthesis of coenzyme A (CoA) which is somewhat different from the usually accepted pathway. It has been considered that pantothenic acid→pantothenylcysteine→pantetheine→4′-P-pantetheine→dephospho CoA→CoA represents the pathway for CoA biosynthesis. Brown studied CoA synthesis in bacterial systems, rat liver, and rat kidney. He obtained convincing evidence that CoA is synthesized by the following pathway: pantothenic acid→4′-

phosphopantothenic acid→4′-phosphopantothenylcysteine→4′-phosphopantetheine→CoA. In addition to enzymatic evidence of this pathway of CoA biosynthesis, evidence for the distribution of these proposed intermediates in tissues was presented. It was found that, while CoA was a major pantothenic acid-containing component of most bacterial and animal tissues, significant quantities of the various proposed intermediates were detected.

Omata (130) found pantothenic acid necessary for growth of the microorganism *Fusobacterium nucleatum*. This requirement could be satisfied by pantothenic acid, pantetheine, or CoA. Barboriak *et al.* (131) found that pantothenic acid deficiency in rats resulted in testicular damage. There was degeneration of the granular and interstitial tissue. Administration of a pantothenic acid antagonist caused an earlier appearance of these lesions and also in a more severe form. Taylor (132) reported that pantothenic acid-deficient rats were more resistant to oxygen poisoning than were normal rats. In this connection, the deficient rats resembled adrenalectomized rats.

Hodges and associates (133, 134) reported additional evidence for the requirement of pantothenic acid by man. Subjects given the pantothenic acid antagonist omega-methylpantothenic acid developed subjective signs considered to be indicative of pantothenic acid deficiency. Although the laboratory tests were not entirely conclusive, the overall appraisal of the data indicates that these subjects were suffering from pantothenic acid deficiency.

Greenberg & Glick (135) studied CoA levels in various regions of the adrenal glands. Injections of ACTH resulted in increases in CoA concentration as did injections of pantothenic acid, and cortisone; hypophysectomy reduced CoA levels. These results are in agreement with many of the clinical signs of pantothenic acid deficiency suggesting adrenal malfunction.

Formica & Brady (136) obtained an extract of pig heart which would enzymatically form malonyl CoA from acetyl CoA and bicarbonate. This reaction represents a step in fatty acid synthesis. McMurray & Lardy (137) studied the possible role of CoA in oxidative phosphorylation. Rat liver mitochondria were subjected to sonic disintegration, centrifuged at 105,000 g, and the washed particles were used to measure oxidative phosphorylation. Addition of heated supernatant extract from the disintegrated mitochondria improved P:O ratios. Addition of CoA to the washed particles greatly increased oxidative phosphorylation and replaced the requirement for the heated extract. Only the reduced form of CoA was active and other sulfhydryl compounds were ineffective. These experiments are interesting and suggest that CoA may function in some manner in the transfer of phosphate from inorganic phosphorus to ATP in the process of oxidative phosphorylation.

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NUCLEIC ACIDS[1,2]

Selected Topics Related to Their Enzymology and Chemistry

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The author has made no attempt at a comprehensive review of the literature of the past year but instead has discussed rather fully a small number of topics. The selections merely represent some of the current interests of the reviewer, and no suggestion is made that the areas covered are necessarily the most exciting or important. On occasions, the reviewer has indulged in criticism and speculation, which have been rationalized on the basis that they provide some slight compensation for the effort expended.

Ribonucleases

Pancreatic ribonuclease.—While the elucidation of the mechanism of action of an enzyme at the molecular level is, at the moment, a goal rather than an accomplishment, the possibility for success seems best for the enzyme pancreatic ribonuclease. The intense efforts devoted to sequence and active site determination have led to a fairly detailed picture of the structure, including information regarding certain essential and unessential residues. The seemingly superhuman task of x-ray crystallographic analysis is being approached with equal zeal, and the day may yet arrive when a complete

[1] The survey of the literature pertaining to this review was concluded in November, 1959.

[2] The following abbreviations are used: DNA for deoxyribonucleic acid; DNase for deoxyribonuclease; RNA for ribonucleic acid; RNase for ribonuclease; A, G, X, U, C, and T for the nucleosides and nucleoside residues possessing, as their constituent bases, adenine, guanine, xanthine, uracil, cytosine, and thymine, respectively.

Polymers formed from ribonucleoside-5′-diphosphates by the action of bacterial polynucleotide phosphorylase are designated poly A, poly G, poly Y, poly C, poly AGUC (made from a mixture of four nucleoside-5′-diphosphates), etc. However, chemically synthesized homopolymers will be referred to in the same manner. Poly dAdT is the polymer formed from the deoxyribonucleoside-5′-triphosphates of adenine and thymine. In representing oligonucleotides a phosphate group is denoted by "p"; when placed to the right of the letter symbol that represents the nucleoside, the phosphate is esterified at carbon 3′ of the nucleoside moiety; when placed to the left of the symbol, esterification is at carbon 5′. Thus ApCp would be a dinucleotide with a monoesterified phosphate on the 3′-carbon of the cytidine residue and ApC would be a dinucleoside monophosphate. Both compounds would have a single 3′,5′-phosphodiester bond. The subscript "x" used as in G_xp would indicate that the nucleoside residue differs in some unknown way from guanosine. Note that this system of terminology is essentially that of the *Journal of Biological Chemistry*.

three-dimensional model of the enzyme can be constructed. The structural requirements for substrates of the enzyme, a subject recently reviewed by Heppel & Rabinowitz (1), can now be more strictly defined. Ukita & Irie (2) have attempted to determine whether the failure of the enzyme to catalyze the hydrolysis of esters of purine 3′-nucleotides is attributable to steric hindrance by the purine residue or to the absence of some functional group found in the pyrimidines. The evidence favors the latter interpretation, for neither the methyl-α-ribofuranoside-2,3-cyclic phosphate nor the methyl-β-ribofuranoside-2,3-cyclic phosphate served as a substrate. It is not surprising then that simple structural analogues of the alkali-sensitive moiety of RNA, such as 2-hydroxyethyl benzyl phosphate and ethylene glycol-1,2 cyclic phosphate, would not be hydrolyzed by the enzyme (3). Witzel (4) has used selective deamination and hydrazinolysis to convert dinucleoside phosphates to derivatives that can be hydrolyzed to 5′-(ribose-3-phosphoryl)ribonucleosides. Thus, GpC after nitrous acid treatment gave XpC, which on mild acid hydrolysis gave 5′-(ribose-3-phosphoryl)cytidine. Compounds of this type were unaffected by RNase and remained so after reduction to the corresponding ribitol derivatives by sodium borohydride (5). On the other hand, reduction of an ester of a pyrimidine-3′-nucleotide to the corresponding 4,5-dihydropyrimidine derivative did not affect its ability to serve as a substrate. However, hydrolysis of the latter to the ester of ureidopropionic acid ribofuranoside-5′-phosphate rendered the diester inert. On the basis of these results, Witzel (5) has postulated that the carbonyl oxygen at C-2 of the pyrimidine ring interacts by hydrogen-bond formation with the sterically well-situated 2′-hydroxyl of its sugar residue (6, 7). This makes the oxygen at C-2′ a better nucleophile and facilitates its attack on the phosphorus of the diesterified phosphate attached to C-3′. This action leads to the formation of the 2′,3′-cyclic phosphate. One might elaborate on this mechanism by suggesting that some essential basic group or center in the RNase molecule initiates the above process by withdrawing entirely or partially (e.g., by hydrogen-bond formation) a proton from the uracil or cytosine residue of the phosphodiester unit under attack. The concerted action could then be represented as in Figure 1.

This mechanism receives some support from the fact that replacement of a hydrogen by a methyl group at N-1 of the uracil residue in the methyl ester of 3′-uridylic acid prevents hydrolysis by ribonuclease (8). It will be of interest to see whether the N^6-acetylcytidine-2′,3′ cyclic phosphate prepared by Michelson (9) is similarly unaffected by the enzyme.

If the above mechanism has some validity, one might expect that other atoms with unshared electron pairs similarly oriented in space with respect to C-2′ might also facilitate hydrolysis. While it is known that 2-thiouracil can replace uracil in RNA without affecting the ability of ribonuclease to attack the adjacent phosphodiester bonds (10), weaker nucleophiles than sulfur might also strengthen the basic properties of the oxygen atom of the 2′-hydroxyl group. Possibly the slow hydrolysis of ApAp and ApUp (11)

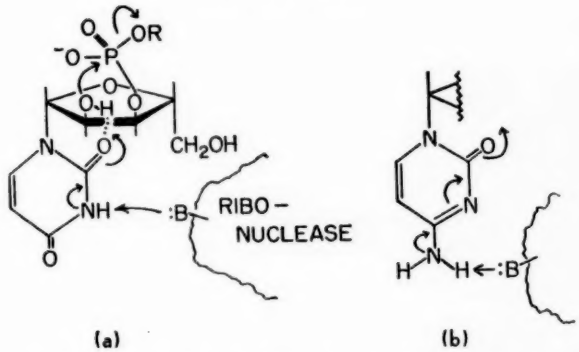


FIG. 1. Proposed mode of action of ribonuclease on (a) an ester of 3′-uridylic acid and (b) an ester of 3′-cytidylic acid.

and polyribosephosphate (12) by high concentrations of RNase can be explained in this way. In the case of the dinucleotides, N-3 of the adenine would be the basic center in proximity to the 2′-hydroxyl of the sugar. In the case of polyribosephosphate, it has been suggested (13) that this role is played by an oxygen atom of a ribose unit of the second chain, which occupies the positions normally taken by purines and pyrimidines and which is bound (ribose to ribose) in 1,1′-glycosidic linkage (12). An alternative explanation for the slow hydrolysis of these unusual substrates would be that the crystalline pancreatic ribonuclease is contaminated by traces of less specific phosphodiesterases. Some support for this theory is derived from the investigations of Takemura and co-workers (14) described below.

The finding (15), now often confirmed (9, 16), that esters of 3′-pseudouridylic acid are hydrolyzed by ribonuclease is also in agreement with the general mechanism proposed by Witzel, since all of the essential structural elements are present (see Figure 2).

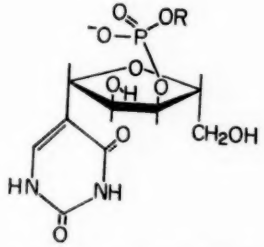


FIG. 2. An ester of 3′-pseudouridylic acid.

Takemura and co-workers (14) have recently reported that riboapyrimidinic acid (17), while slowly hydrolyzed by commercial crystalline ribonuclease, was much more resistant to the ribonucleases A and B obtained by the fractionation method of Hirs, Moore & Stein (18). It was suggested that the unfractionated enzyme probably contains, in addition to RNase A and RNase B, a small amount of another ribonuclease, which hydrolyzes purine 3′-nucleotide esters, or a non-specific phosphodiesterase. Several objections to this experiment might be raised. First, the riboapyrimidinic acids, which are prepared by hydrazinolysis of yeast RNA, are as yet very poorly defined chemically. Secondly, as the authors state, the resistance of the substrates to the action of the enzyme increases with decreasing pyrimidine content, and with normal enzyme to substrate ratios only slight hydrolysis is observed for the preparation which is almost free of pyrimidines. Lastly, inasmuch as extensive reaction was observed only at very high enzyme to substrate ratios (1:2 and 5:1), it should be ascertained whether the degradation of the apyrimidinic acid is caused by enzyme catalysis or by non-specific base-catalyzed β-elimination (19, 20).

Related to this is the claim of Hakim (21, 22, 23), as yet unconfirmed, that crystalline ribonuclease can be resolved by two-dimensional paper electrophoresis into four active fractions. Each of these active fractions has preferential specificity for one of the four common ribonucleoside-2′,3′-cyclic phosphates but is not completely without action on the others. Attempts to resolve ribonuclease A and B by the same procedure failed, and one can only suggest that this very provocative result be investigated by others in the field.

Substitution of a bromine atom or a methyl group for a hydrogen atom at the 5-position of uracil in a 2′,3′-cyclic ribonucleotide does not alter the ability of the compound to serve as a substrate of the enzyme (24). This is in accord with the finding that polyribothymidylic acid (25) and the cyclic thymine ribonucleotide (16) can be degraded by ribonuclease to mononucleotides.

Since the procedure of Michelson (26) for polynucleotide synthesis gives both 2′,5′- and 3′,5′-phosphodiester linkages in about equal amounts, the poly U, poly C, and polypseudo U thus prepared can be broken down by ribonuclease to oligonucleotides possessing exclusively 2′,5′-linkages (9, 26).

Using pancreatic ribonuclease, Reddi (27) has digested the nucleic acids isolated from three different strains of tobacco mosaic virus and estimated the amounts of four particular oligonucleotides in each. He has concluded from the differences found that the nucleotide sequence cannot be identical in the three strains. Reddi (27) also reported the absence of the dinucleotide GpCp in the digests examined. On the other hand, Rushizky & Knight (28), using a two-dimensional mapping procedure, have succeeded in separating and identifying all of the major products obtained by digestion of tobacco mosaic virus-RNA with ribonuclease, among which was GpCp. This has recently been confirmed by an independent investigator (29).

Kalnitsky & co-workers (30, 31, 32) have presented evidence indicating that the denaturation of yeast ribonucleic acid by urea renders it more susceptible to ribonuclease. They suggest that in disrupting the secondary structure of the RNA, the urea effectively increases the substrate concentration by presenting a greater number of hydrolyzable groups to the enzyme. The picture, however, is clouded by the fact that concentrated urea was found both to stimulate and to inhibit the action of ribonuclease upon RNA, depending upon the assay procedure used. Since the possibility exists for modifying the secondary structures of the substrate or of the enzyme, or of both simultaneously, the results are difficult to interpret. Since no absolute correlation between rate of acid liberated (bonds broken) during digestion and configuration of substrate was made, it seems premature to draw the parallel between this effect and the classical effect of denaturation upon enzymatic digestion of protein (33).

Ribonucleases of Aspergillus oryzae.—Sato & Egami (34) have furnished evidence for the existence of two thermostable ribonucleases in taka-diastase, a commercial product obtained from an extract of *Aspergillus oryzae*. The enzymes, which after preliminary purification steps can be clearly separated from one another by zone electrophoresis on starch, have been designated T_1 (major component) and T_2 (minor component). They differ from one another with respect to their pH optima and specificities. Ribonuclease T_1 has been most extensively purified and, on the basis of sedimentation and electrophoretic studies, is believed to be almost homogeneous and certainly free of T_2. More recently, Sato-Asano (formerly Sato) (35) has identified the mononucleotides and terminal groups of oligonucleotides produced when yeast RNA is the substrate. After extensive digestion, only guanosine-3′-phosphate or oligonucleotides terminating in guanosine-3′-phosphate were found. The corresponding cyclic phosphate derivatives were shown to be intermediates. Thus, T_1 appears to be specific for the esters of guanosine-3′-phosphate, a fact clearly demonstrated by the ease with which it hydrolyzes the guanine-rich oligonucleotides of the "core" material remaining after the digestion of RNA with pancreatic ribonuclease. Like the pancreatic enzyme, T_1 has no metal ion requirement and, in fact, is activated by ethylenediamine tetraacetic acid and inhibited by certain divalent cations, including Mg^{++} and Ca^{++} (34). T_2 at its present stage of purity liberates adenosine-3′-phosphate and the pyrimidine-3′-mononucleotides early in the digestion of RNA and liberates guanosine-3′-phosphate only at a later stage. Further proof that the two enzymes have some specificity for esters of purine 3′-nucleotides is provided by the investigations of Takemura & Miyazaki (36). They have shown that riboapyrimidinic acid (17) serves as a substrate for either enzyme. For equal substrate to enzyme ratios, the fraction of the total phosphorus released as acid-soluble phosphorus per unit time was roughly equivalent for RNA itself and for RNA that had been depyrimidinated by 15 to 30 per cent and by 90 to 100 per cent. Both T_1 and, with further purification, T_2 should be very useful for structural studies of viral and "soluble" RNAs.

Leaf ribonuclease.—Reddi (37), continuing his studies on leaf ribonuclease first described by Holden & Pirie (38), has shown that poly U is hydrolyzed at a more rapid rate than poly C. There is a greater than fivefold difference in the initial rates, a result in accord with his earlier findings (39). A correlation between leaf ribonuclease activity and the extent of tobacco mosaic virus synthesis has been demonstrated by the same investigator (40). The older bottom leaves contained more enzyme and produced a greater amount of virus on infection than did the younger top leaves. It was suggested that the role of the leaf ribonuclease in the infected leaf may be the breakdown of leaf RNA and the synthesis of viral RNA by a process of rearrangement. Since it is generally recognized that the reactions by which biopolymers are synthesized are not the reverse of the processes by which they are degraded, the suggestion is somewhat surprising.

Intracellular ribonucleases.—Recent advances in the purification and characterization of intracellular ribonucleases were discussed at a recent conference on "Enzymes of Polynucleotide Metabolism" (41 to 45). The localization, partial purification, and characterization of some of the enzymes found in pancreas (42), spleen (43), and liver (45) were described.

Micrococcal Nuclease

Improved methods for the purification of this enzyme, which was originally discovered by Cunningham *et al.* (46) in the culture medium of *Staphylococcus aureus*, have recently been described by Dirksen (47), Reddi (48), and Privat de Garilhe and co-workers (49). Unfortunately, each of these workers has used a different assay system, and no comparison can be made of the activities of the preparations. While there can be no certainty that the hydrolysis of RNA and DNA by these preparations is the result of a single enzyme, the similarities between the deoxyribonuclease and the ribonuclease activities are numerous and support the concept of one enzyme. The similarities include the high stability of the enzyme, the pH optimum, the calcium ion requirement, the position of phosphate attachment in the products, and the distribution of products (50). The report of Privat de Garilhe and coworkers (49) that ribonuclease activity was removed by chromatography on IRC-50 appears to be based on a faulty assay procedure for ribonuclease, and attempts to confirm this finding have been unsuccessful in the author's laboratory and elsewhere (51). The products with either substrate are mono-, di-, tri-, and possibly larger oligonucleotides bearing a 3′-monoesterified phosphate (29, 50, 52, 53). When, however, the synthetic polyribonucleotides poly A (29, 50), poly U (48), or poly C (29, 48) or the synthetic two-stranded polydeoxyribonucleotide, poly dAdT (50), is employed as a substrate, only mono- and dinucleotides are present in the digests. Biosynthetic poly G was not available for testing, but a chemically synthesized poly G (26) possessing both 2′,5′- and 3′,5′-phosphodiester linkages proved resistant to hydrolysis (54). On the other hand, a poly A prepared by Michelson (26) and also possessing both types of linkage was partially hydrolyzed (54). The re-

sistance of bonds adjacent to guanine residues may explain the occurrence of fragments larger than dinucleotides in digests of RNA (29, 50), DNA (50, 53), and poly AGUC (29). It should be mentioned that Reddi is not in complete agreement with the above views, since he finds mono- and dinucleotides exclusively in digests of tobacco mosaic virus-RNA (48, 55).

Further elucidation of the specificity has come from studies carried out in the author's laboratory. Dirksen & Dekker (56, 57) found that the enzyme attacked native and heat-denatured DNA at different rates and that, in the appropriate ionic environment, the heat-denatured substrate was hydrolyzed at more than 100 times the rate observed for the native substrate. The data of Marmur & Doty (58) indicate that those double-helical DNA molecules (and presumably, segments of molecules) possessing high concentrations of deoxyadenylic acid and thymidylic acid residues are more readily disrupted by heat than those possessing lower concentrations. It was therefore of interest to find that mono-, di-, and trinucleotides possessing A or T residues, or both, were liberated more rapidly during digestion than those possessing C or G residues, or both (53). This "AT-preference" is also apparent on re-examining the data of Cunningham (50) on the nature of the products resulting from the action of the enzyme on DNA. All of the major dinucleotide components have either A or T on the terminal residue that bears the free 5′-hydroxyl group. Moreover, 3′-deoxyadenylic and 3′-deoxythymidylic acids are the major mononucleotide components with 3′-deoxycytidylic occurring in a somewhat lesser amount and 3′-deoxyguanylic in very small amount. This has been explained (59) on the basis of a very high degree of "AT-preference" during the initial stages of digestion when the specific G-C base pairing impedes the progress of the enzyme. In the later stages of digestion, when single-stranded polynucleotides are present in larger amount, the specificity becomes broader and G- and C-containing fragments begin to accumulate. A similar "AU-preference" can be observed in the case of RNA digestion (48, 50, 55); however, the specificity is not as marked, presumably because of the single-strand nature of the substrate. In keeping with this theory of specificity is the finding that the double-stranded poly dAdT ("A-T" polymer) (60) is much more rapidly hydrolyzed by the enzyme than is native DNA (59).

Dirksen & Dekker (57) have also reported that a 63 per cent hyperchromic effect, almost the theoretical maximum, is observed when one digests native DNA with the micrococcal enzyme. This undoubtedly is attributable to the high percentage of total bonds broken (60 to 70 per cent).

On the basis of its action on tobacco mosaic virus-RNA and the biosynthetic polymers, poly U and poly C, Reddi (48) has postulated that the enzyme first cleaves the large polymer internally to produce several polynucleotides, which in turn are broken down by stepwise cleavage of 3′-nucleotide units until reduced to the size of dinucleotides. While his original picture of this process implied that the stepwise removal proceeded from the end of the polynucleotide that bears the free 5′-hydroxyl group, a later

communication offers evidence for attack from the other end (61). This is in accord with the results of Dirksen (62) and Roberts & Dekker (63), who have investigated the action of the micrococcal enzyme on several oligoribonucleotides. Substances such as ApApUp, ApApCp, and GpGpUp gave as ultimate products ApAp or GpGp and the pyrimidine-containing mononucleotides. While the action of the enzyme on these particular substrates can be explained in terms of an exoesterase attack from the 3′-phosphate end, many more oligonucleotide substrates will have to be tested to ascertain whether a unified mode of attack exists.

Finally, it should be emphasized that sequence determination in nucleic acids will be a task of much greater complexity than that encountered with proteins because of the smaller number of different monomeric units. The main hope for success appears to lie in the discovery of enzymes that will cleave only at those loci where a specific sequence of three or four nucleotide residues are found. The polynucleotides resulting from this type of action might then be sequentially analyzed by stepwise removal of nucleotide residues with an exophosphodiesterase such as that obtained from snake venom (64). The results with micrococcal nuclease are encouraging in this respect since they indicate that sites of initial attack may be determined, at least in part, by several nucleotides in sequence. Moreover, this "configurational" specificity, which permits the enzyme to select between amorphous and crystalline regions or easily or difficulty denaturable regions in a polymer, should permit the use of the enzyme as a structural probe, as has been suggested recently for several proteolytic enzymes (65).

Minor Constituents of Nucleic Acids

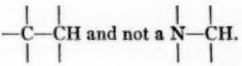
While only 5-methylcytosine, 5-hydroxymethylcytosine, and 6-methylaminopurine have been found (see previous reviews) in addition to the four common bases in DNA, the list of unusual constituents in RNA continues to grow. The occurrence of methylated adenines, reported in last year's review, has been more rigorously established with the isolation of the ribosides and ribotides of these compounds from several ribonucleic acids and their identification by chromatographic, electrophoretic, and spectroscopic methods (66). The naturally occurring nucleosides were compared with authentic samples prepared both chemically and enzymatically. Evidence has been presented to show that all three—2-methyladenine, 6-methylaminopurine, and 6,6-dimethylaminopurine—as well as thymine, are present in RNA as nucleotides linked by 3′,5′-phosphodiester linkages. A new guanine derivative, 2,2-dimethylamino-6-hydroxypurine (67), has been added to the existing list, which included 1-methylguanine and 2-methylamino-6-hydroxypurine. All three compounds have been isolated as their nucleosides, and evidence for their structures and their existence as true components of RNA has been presented, as well as for the amounts found in different sources (67, 68). Although the methylated bases were found in most of the microorganisms examined, as well as in some plant and animal sources, none were found in the RNA of tobacco mosaic virus or in that of turnip-yellow mosaic virus.

A new and as yet unidentified substance, isolated from wheat germ RNA (69), has an absorption peak at 335 mμ (one of two), which shifts into the visible region of the spectrum upon raising the pH to 12.

By far the most interesting of the new components, chemically, is the substance variously identified as the "fifth nucleotide" (15), "nucleotide W" (70), or pseudouridylic acid (71). Evidence that the nucleoside derived therefrom is 5-ribofuranosyluracil has now appeared (72 to 75), and the only structural feature yet unsolved is the configuration about the glycosyl-carbon linkage. The resistance of this latter linkage to conventional hydrolytic procedures was a major deterrent to structural investigation. Yu & Allen (73) ultimately found that periodate oxidation followed by treatment with bromine gave uracil and 5-bromouracil from both uridine and the unknown nucleoside. They also reported, as did Cohn (72), that the absorption spectra at pH 7 and pH 12 resembled that of 5-hydroxymethyluracil. Scannell, Crestfield & Allen (74) concluded from methylation studies on the nucleoside that the 1, 2, 3, and 6 positions were not involved in glycosyl linkage and that C-4 was a less likely position for sugar attachment than C-5. Cohn (75) identified the corresponding nucleotide as the same material that he had observed, as early as 1951, as an unknown peak in ion-exchange elution patterns of RNA digests (76). He has presented very convincing evidence for the 5-ribosyluracil formulation for the nucleoside, the best of which is the following:

(*a*) Periodate oxidation followed by borohydride reduction gives an unstable ether that after hydrolysis will react with the same two reagents to give a substance chromatographically and spectrally identical to 5-hydroxymethyluracil.

(*b*) The nuclear magnetic resonance spectrum, when compared with those of uridine, thymine, 4-methyluracil, 5-hydroxymethyluracil, and 5-hydroxyuridine, demonstrated, among other things, that there is a proton on C-4 but not on C-5 and that the proton on C-1′ is similar to a



The chemical behavior of 5-ribosyluracil is unusual in that strong acid hydrolysis gives two additional nucleosides, presumably the ribopyranosyluracil and the corresponding open chain compound. Moreover, reduction of the nucleoside gives a substance tentatively identified as 5-ribityluracil, indicating that C-1′ of the new nucleoside may have a reactivity similar to that of the methylene carbon of a benzyl ether.

As has been pointed out (75), the unusual structure and reactivity of 5-ribosyluracil raises many questions regarding its biosynthesis, catabolism, and function in the polynucleotide structure. It should be remembered that standard methods of determining base composition of nucleic acids do not liberate uracil from the new compound.

Studies on the distribution of the minor constituents of RNA have led to one of the more exciting findings of the past year, namely, that these sub-

stances are found primarily in the soluble RNA, an important factor in protein synthesis (68, 77, 78). Since separate functions have been suggested for the microsomal and soluble RNA fractions of rat liver, Dunn (77) has compared the relative proportions of the additional base components from these two sources. His data are reproduced in Table I. With a similar objective, Bergquist & Matthews (78) have determined the distribution of methylated purines in mouse liver and a mouse adenocarcinoma. They have also found that the "soluble" cytoplasmic fraction from these two sources contains substantially more of these bases than the mitochondrial or microsomal fractions. Moreover, the soluble RNA from tumor, particularly young tumor, is richer in these bases than is that from liver. Two explanations have been offered for the small amounts of these components found in the microsomal

TABLE I

RELATIVE PROPORTIONS OF ADDITIONAL COMPONENTS IN RNA FROM RAT LIVER MICROSOMES AND SOLUBLE FRACTIONS*

Component	Microsomes†	Soluble†
Pseudouridine	7.5	25
5-Methylcytosine	0.4	10
6-Methylaminopurine	0.5	8.1
6,6-Dimethylaminopurine	0.1	0.1
1-Methylguanine	0.1	3.3
2-Methylamino-6-hydroxypurine	0.1	2.3
2,2-Dimethylamino-6-hydroxypurine	0.1	3.0

* See (77).

† Values are in moles per 100 moles uridine.

RNA. The first suggests that the microsomes contain a proportion of soluble RNA in addition to an RNA in which these unusual bases are absent. The latter might be functionally similar to the RNA of plant viruses that are free of the additional components. One would then have to conclude that the various RNAs found in the soluble fraction are present in the microsomal fraction in different amounts (77). The second suggests that the microsomal fraction, as well as the mitochondrial fraction, is contaminated with small amounts of soluble RNA (78). Only further purification of the functional microsomal fraction will enable a differentiation between the two possibilities.

One of the consequences of methyl substitution of the purine bases in nucleic acids would be a diminished opportunity for base pairing in the specific manner proposed by Watson & Crick (78a). It has been suggested that this should give the soluble RNAs more "single strand" character, which might be in keeping with their role as specific transferring agents for amino acids. Physical chemical studies (79), however, have failed to substantiate this view and have indicated instead that soluble RNA has secondary struc-

ture to about the same extent as tobacco mosaic virus-RNA, in which no unusual base components have been found. Alternatively, the methylated purines may provide alterations in the template that are necessary for coding a less common amino acid (66).

Davis and co-workers (16) have found four minor nucleotides, in addition to pseudouridylic acid, in those RNAs of yeast that are soluble in 1 *M* NaCl (15, 80). Three of these have been identified as the ribonucleotides of thymine, 6-methylaminopurine, and 1-methylguanine. The fourth appears to be a modified guanine nucleotide. An interesting finding of this group was the small amount (10 to 20 per cent) of thymine ribotide released by ribonuclease digestion of these RNAs compared with the amount of uridylic, cytidylic, or pseudouridylic acid released (45 to 55 per cent). They have suggested that the bulk of the thymine riboside-5′-phosphate residues may be attached to the 3′-positions of purine nucleotide residues in this RNA. In view of their similarity with respect to the amount of minor nucleotide components and their low molecular weight, it has been suggested (77, 81) that the yeast RNAs soluble in 1 *M* NaCl are functionally similar to the "soluble" RNAs of animal tissues. Otaka, Hotta & Osawa (81) have fractionated the subcellular components of yeast by differential centrifugation and have analyzed the various fractions for pseudouridylic acid. While the cell walls, large granules, and 80 S particles had only traces of this nucleotide, the supernatant fraction had an appreciable amount (14 per cent of the adenylic acid).

Isolated reports have appeared of new purine bases or nucleosides that are found as such, rather than as components of polynucleotides. Thus, a purine base with the empirical formula $C_6H_7N_5$ has been found in extracts of the giant siliceous sponge, *Geodia gigas* (82), and succinyladenosine has been found in human cerebrospinal fluid (83). The latter compound may have been derived from the corresponding nucleotide known to be involved in purine interconversions in microbial systems (84).

Hydrolysis of Nucleosides

Kenner (85) has contributed a very enlightening discussion on the mechanism of acid hydrolysis of glycosylamines, particularly of nucleosides. He makes three important points: (*a*) for hydrolysis to occur (at an appreciable rate), the proton must become attached to the ring oxygen of the glycosylamine [see Fruch & Isbell (86)]; (*b*) when the first proton adds to a nitrogen atom of a heterocyclic base, hydrolysis will occur if ready transfer of the proton to the ring oxygen of the sugar can be achieved; and (*c*) if transfer of the proton cannot take place as in (*b*), hydrolysis can still occur if the charge is so widely distributed that there is a reasonable chance of adding a second proton. Kenner states that the stability of the glycosyl derivatives of pyrimidines, purines, imidazoles, and various other amines fits this general theory. We suggest that Kenner's second point should receive stronger emphasis. Using molecular models, it is easily demonstrated that N-3 of the purine ring, an amino substituent at C-2 of the pyrimidine ring, and an amino substituent at C-5 of the imidazole ring can all lie in close proximity to the sugar ring

oxygen, thus facilitating transfer of a proton. While protonation at the above nitrogen sites may not be favored in all cases, the necessary tautomeric forms of the cation are undoubtedly present to some extent in the equilibrium mixture and may well be the reactive intermediates in hydrolysis. It has now been found that isocytidine, recently prepared by the action of methanolic ammonia on a derivative of O(2), 5′-cyclouridine (87), is much more readily hydrolyzed than cytidine (88). Since both compounds have an equal number of nitrogen atoms over which the charge of the protonated form can be distributed, the facile hydrolysis of the isocytidine has been attributed (88) to the superimposition of the amino nitrogen on the ring oxygen of the sugar. Proton transfer could thus take place readily (see Figure 3). Similarly, ribosyl imidazoles are much more readily hydrolyzed if they possess a 5-amino substituent (89). The stability of cytidine would imply that the carbonyl oxygen at C-2 is an unlikely site for proton addition and that the cationic forms are almost completely restricted to those in which the proton resides on a nitrogen (Figure 3). The disposition of the nitrogens relative to the oxygen of the sugar ring precludes transfer in all cases. This is in agreement with the spectrophotometric argument for the locus of proton addition to cytidine presented below.

Sites of Proton Addition to Pyrimidines and Purines

While students of heterocyclic chemistry have long agreed that the protonation of a 2- or 6-amino-substituted purine or pyrimidine occurs at a ring nitrogen rather than at the exocyclic nitrogen [see Mason (90)], biochemists have been reluctant to accept this fact. It might be well, therefore, to state briefly the arguments for a ring location: (*a*) Protonation of a ring nitrogen, such as N-7 or N-3 in adenine and guanine and N-1 in adenine and cytosine, results in resonance stabilization, since two or more mesomeric forms are possible, see Figure 3b. On the other hand, only one structure can be written for the N-2 protonated guanine and one each for the N-6 protonated adenine and cytosine. (*b*) Spectrophotometric comparison of the protonated base with the cationic forms of suitably methylated derivatives usually shows a similarity between the

$$=\overset{|}{\underset{\oplus}{N}}H \text{ and a particular } =\overset{|}{\underset{\oplus}{N}}-CH_3.$$

This comparison can be made because the methyl group is optically transparent in the ultraviolet and normally causes only a slight shift in λ_{max} when substituted for a hydrogen. On the other hand, protonation of an exocyclic amino group will destroy the π-p conjugation between the unshared p-electrons of the amino nitrogen and the π system of the ring. This will affect the spectrum dramatically, as can be shown with aniline: the free base has an intense band at 251 mμ while the anilinium ion has relatively weak absorption similar to benzene (91). (*c*) When model compounds such as 2-amino-

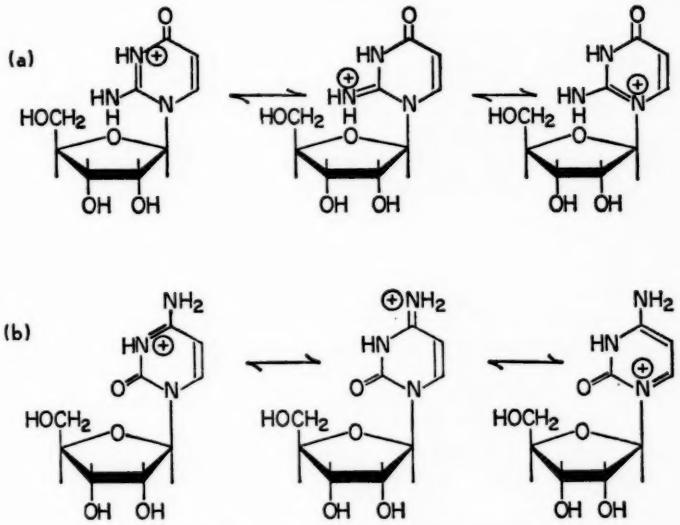


FIG. 3. Examples of resonance stablization.
(a) Isocytidine (b) Cytidine
Also effect of site of proton addition on ease of hydrolysis.
(a) More easily hydrolyzed (b) More difficulty hydrolyzed

pyridine or 2-aminopyrimidine are treated with alkylating agents such as methyl iodide or dimethyl sulfate, the alkyl group goes preferentially to a ring nitrogen (92). Since alkylation is assumed to occur at the site of highest electron density, one might assume that protonation proceeds in an analogous manner. However, chemical reactivities are determined by a large number of factors, and this is by far the weakest of the three arguments.

Pullman (93), in reply to criticism by Lynch, Robins & Cheng (94), states that the basicity of ring nitrogens does not depend solely upon the formal electrical charge on these atoms but upon other factors as well (95). He concludes that N-1 is the most basic site in adenine and N-7 in guanine. As supporting evidence he cites the formation of adenine-1-oxide (96) and the preferential reaction of N-1 of adenine (97) and N-7 of guanine to alkylating agents (98, 99). Reiner & Zamenhof (100), however, have found that the N-7 position in both adenine and guanine is the most reactive to dimethyl sulfate irrespective of whether a free base or highly polymerized DNA is methylated. While the cationic forms of 7-methylguanine and guanine (100), as well as the cationic forms of 7-methyl deoxyguanosine-5′-phosphate and deoxyguanosine-5′-phosphate show marked similarity in ultraviolet spectra (98), one cannot absolutely exclude N-3 as the site of protonation since the

corresponding N-3 methylated guanine is not available for spectral examination. However, using the spectral argument, N-6 can be eliminated as a possible site, since the proton would occupy the unshared electron pair of the amino group and prevent it from resonating with the conjugated system of the pyrimidine ring of guanine (90). This would have the effect of changing the spectrum to one approximating that of 6-hydroxypurine (90), but this change is not observed. At the moment, then, we must conclude that N-7 is the most likely site for the attachment of the first proton and that pK_a 3.3 of guanine refers to the pH of half-dissociation at this position.

Since neither the N-7 nor the N-3 (101) methylated adenines have a spectrum similar to that of adenine in acid solution, the N-7 and N-3 protonated forms of adenine are probably not present in sufficient amount to make a major contribution to the spectrum. N-1 is known to bear the proton in solid adenine hydrochloride (102), and, although N-1 has been suggested as the most reasonable position for the initial protonation of adenine in solution on the basis of resonance stabilization (103) and on indirect arguments related to the stability of the double-stranded helical model of poly A (104), it has not been possible to confirm this by the spectral method because of the unavailability of the appropriate methylated derivative.

Assignment of proton location in the cationic form of cytidine can be made with reasonable confidence. The nucleoside rather than the free base is considered because of the availability of suitable model compounds. Here again the spectral argument can be used to eliminate possible protonation at N-6 or N-3. On the basis of resonance stabilization, however, both N-1 and O-2 would appear as likely sites. One can distinguish between the two by comparing the spectra in acid solution of cytidine, dimethylcytosine (105), and O(2), 2′-cyclocytidine (106) (see Figure 4). It is found that the spectrum of cytidine (λ_{max} at 280 mμ and 212 mμ) is similar to that of 1,3-dimethylcytosine (λ_{max} at 281 mμ and 212 mμ) and different from that of O(2),2′-cyclocytidine (λ_{max} at 262 mμ and 232 mμ). While it might be argued that O(2),2′-cyclocytidine is a very special form of an O-2 substituted cytidine and might therefore have an unusual spectrum, studies on the structurally similar O(2),2′-cyclouridine (87) show that it has a spectrum resembling that of O(2)-methyluridine. Thus the comparison appears valid. It should be mentioned that the carbonyl oxygen of cytosine has been suggested as the site of initial protonation during the low-temperature titration of DNA with acid (107). While the evidence for protonation on cytosine is excellent, the location of the hydrogen ion on O-2 is contrary to the spectral evidence cited above.

Detailed knowledge of preferred proton-acceptance sites is necessary if we are to interpret properly the results of studies of the titration of nucleic acids with acid (108, 109, 110). Moreover, this information should lead to a better understanding of acid-induced configurational changes in polynucleotides.

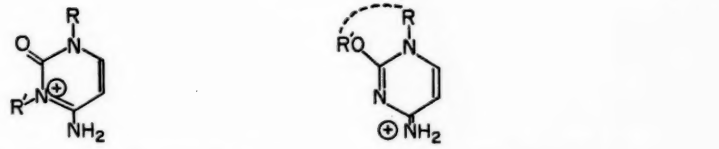


FIG. 4. Possible protonated forms of cytidine and structural analogues.

CHEMICAL HYDROLYSIS OF RNA

There are a number of reports in the earlier literature which indicate that the bonds in RNA differ in respect to their stability to alkaline hydrolysis (111). The evidence from titration (112), x-ray studies on crystalline ribonucleoproteins such as tobacco mosaic virus (113, 114), and degradation by specific enzymes would appear to rule out branching or any other unusual form of phosphodiester bond. Thus the variation in stability is most likely attributable to environmental factors (steric, electrostatic, etc.) which modify the ease of attack of the alkoxide ion (at C-2′) on the phosphorus atom of the adjacent phosphodiester bond. Related to this are the frequent reports that alkaline hydrolysates of RNA contain ultraviolet-absorbing components other than mononucleotides.

Kemp & Allen (115), in agreement with earlier findings from the same laboratory, observed that 3 per cent of the total phosphorus appeared as oligonucleotides under conditions generally assumed to give 100 per cent hydrolysis. Potter & Dounce (116) also found, by ion-exchange chromatography, a fraction eluted after the mononucleotides that was rich in adenine and guanine. This subject has been reviewed recently by Lane & Butler (11), who by their own studies have considerably clarified the nature of the alkaline hydrolysis of RNA. Using a combination of ion-exchange and paper chromatography, these authors isolated and identified from an 18 hr. digest of RNA in 1 *M* KOH, the dinucleotides ApAp, ApGp, ApUp, GpAp, and GpCp[3] (117). Although all of these compounds could be hydrolyzed by more extensive treatment with KOH, it was found that the first order rate constants for their alkaline hydrolysis were appreciably lower than those of the dinucleotides UpUp and CpCp not found in the 18 hr. hydrolysate. For the two extremes, UpUp and ApAp, the rate constants varied by a factor of ten. A very similar study has been reported by Dimroth & Witzel (118), who have isolated all of the 16 possible dinucleoside monophosphates after bismuth hydroxide-catalyzed hydrolysis of RNA (119). Using 0.1 *N* NaOH, at 25°, they found that CpC and CpA were almost completely hydrolyzed in $2\frac{1}{2}$

[3] The terminal phosphate can be on either the 2′ or 3′ position.

hr., while ApC, ApG and GpA required 5 hr. for an equivalent degree of hydrolysis. Moreover, in a 100 min. digest of yeast RNA by bismuth hydroxide, they found a predominance of those dinucleoside monophosphates of the Pu-p-Z type, where Z may be purine or pyrimidine. In certain respects, the bismuth hydroxide-catalyzed reaction, which is conducted at pH 4, is akin to acid-catalyzed hydrolysis in that the rearrangement of a small percentage of the 3′,5′-phosphodiester bonds occurs to give the corresponding compounds with 2′,5′-bonds (119, 120). Thus, direct comparison with the results of an alkaline hydrolysis may not be valid.

Lane & Butler (117) have pointed out that those bonds most resistant to alkali are in every case the bonds that are resistant to pancreatic ribonuclease. The parallelism is most striking in the case of a 2 hr. hydrolysate, in which they found that all the dinucleotides that are not susceptible to digestion by pancreatic ribonuclease (i.e., those of the Pu-p-Py-p and Pu-p-Pu-p type) were found in roughly equal amount and comprised 80 to 90 per cent of the dinucleotides present (11). This recalls the early work of Magasanik & Chargaff (121), who found that during alkaline hydrolysis (pH 13.5, 30°), uridylic acid was set free most rapidly, guanylic acid more slowly, and adenylic acid at one-half the rate of guanylic acid. The recent confirmation and expansion of these early results leads to the conclusion that alkaline hydrolysis may be far more specific than anticipated and that controlled nonenzymatic hydrolysis should be further explored as a method of cleaving long polyribonucleotide chains into discrete units of a size amenable to sequence and end-group determination.

Finally, it might be asked whether the variation in alkali stability of the phosphodiester bonds of dinucleoside phosphates, dinucleotides, and polynucleotides reflects some fundamental configurational difference in addition to a purine-pyrimidine difference. It is known from the work of Dimroth & Witzel (118) that, among the dinucleoside phosphates, those with a pyrimidine-nucleoside residue attached to the diesterified phosphate at C-3′ are more readily eluted from ion-exchange columns than those with a purine-nucleoside residue. For example, CpA is eluted prior to ApC. They suggest that this is more likely caused by a difference in the size of the hydrated molecules (ApC is larger) than by a difference in charge (or pK), since CpA also travels farther on paper electrophoresis at pH 7.0 than ApC. Such differences in size might be the result of base-base interaction since these authors have observed hypochromicity in these simple substances, in agreement with the results of Privat de Garilhe & Laskowski (122), Sinsheimer (123), and Michelson (26).

Michelson, who has recently synthesized a large number of isomeric dinucleoside monophosphates differing both in sequence and in the type of phosphodiester linkage (3′,5′ or 2′,5′), has also presented evidence for configurational differences (9). For example, 2′,5′-linked compounds have a higher electrophoretic mobility than the corresponding 3′,5′-linked compounds, which is paralleled in each case by a greater alkaline hyperchromicity for the 2′,5′-compound. Michelson interprets this as meaning that a

stronger interaction is possible between the bases of the 2′,5′-linked compounds, thus restricting rotation and giving more compact or "streamlined" molecules. Although there is some indication that similar differences in compactness might exist between pairs of isomers that differ only in sequence, insufficient electrophoretic data are available to make a generalization. As a practical point, it should be emphasized that the electrophoretic and chromatographic mobility of a dinucleoside monophosphate, dinucleotide, or trinucleotide will be largely determined by the nature of the base of the terminal residue bearing the free 5′-hydroxyl group, because this group is farthest removed from a phosphate group (124) and, thus, will be most exposed for reaction with solvent or chromatographic medium (paper or resin). This can readily be seen in the two-dimensional mapping procedure of Rushizky & Knight (28), in which the position of an oligonucleotide in a ribonuclease digest of RNA is determined by the terminal residue bearing the free 5′-hydroxyl group. The dependency on this single factor is so great that one can make reasonable predictions regarding the sequences of tri- and tetranucleotides solely on the basis of their location on the map. In the light of this fact, a simpler alternative can be proposed for the slower elution from ion-exchange columns and slower electrophoretic migration of ApC when compared with CpA. In the case of ApC, the adenine moiety will be farthest from the phosphate, and the diester will behave more like a derivative of adenylic acid than of cytidylic acid. By the same token, CpA would behave more like a derivative of cytidylic acid. Since purine nucleotides are known to be "adsorbed" more strongly to ion-exchange resins (125) and filter paper (126) than the corresponding pyrimidine derivatives, the result observed would be in agreement with theory.

Although the slow rate of hydrolysis accounts for much of the non-mononucleotide material in alkaline digests of RNA, it does not account for all. Both Smith & Dunn (127) and Lane & Butler (11) have reported the isolation and partial characterization of substances that are larger than mononucleotides and completely stable to alkali. The former (127) have tentatively identified 12 alkali-stable dinucleotides from several sources, including wheat embryo, plant leaves, and the microsomes and soluble fraction of rat liver. Two of the dinucleotides, G_xpUp and U_xpGp, were enzymatically converted to dinucleoside monophosphates and degraded by the periodate-alkali procedure (128, 129) to substituted nucleotides (G_xp and U_xp). Dephosphorylation gave periodate-resistant nucleosides, the sugar component of which proved identical in its chromatographic behavior to 2′- (or 3′-) O-methylribose. The final identification of these substances and information regarding their metabolic origin and functional importance will be awaited with interest.

The Interaction of Nucleic Acids with Ions

It is of biological and physical chemical interest to ascertain whether nucleic acids exist in isotonic or other solutions as fully charged or as essentially uncharged molecules, i.e., whether the net charge on a molecule is that

which one would calculate by summating the phosphodiester groups in the molecule or some small fraction of this. The evidence from studies performed prior to 1957, ably summarized by Shooter (130), would indicate that at moderate ionic strengths sodium ions are bound to up to 90 per cent of the phosphate groups. This conclusion, which was based primarily on titration and mobility data, has been challenged by Zubay & Doty (131). The latter maintain that the sodium ions are not bound to any specific sites in DNA but that a portion of these *gegen*ions (*ca.* 50 per cent) remain in close proximity to the polyanion as a result of potential binding. On the other hand, they report that their conductimetric and equilibrium dialysis experiments show that magnesium ions are lightly bound to DNA even in the presence of 0.2 *M* NaCl. Upon denaturation, the binding is enhanced and thermally denatured DNA becomes saturated at about 0.7 equivalent Mg^{++} per nucleotide residue. Cupric ion was bound more tightly to undenatured DNA than Mg^{++}, causing marked aggregation as determined by the fall in viscosity. At moderately high concentrations of Cu^{++}, precipitation ensues.

Accepting the stronger binding of divalent metals to denatured DNA, Zubay (132) has suggested that the actual sites of binding may be the adenine and guanine residues, the binding energy being exaggerated because of the electrostatic field created by the phosphate groups. He points out that, when not in the specifically hydrogen-bonded form of native DNA, the purines have a favorable site for metal chelation involving N-7 and N-6 (or O-6). The fact that formaldehyde reduces the binding by about 60 per cent is presented as supporting evidence, since it is believed that formaldehyde reacts with the amino groups of adenine (133).

Felsenfeld & Huang (134), using conductimetric titration, have studied the interaction with divalent cations of the synthetic polynucleotides, poly A and poly U. The titration curves were the same for both polymers, which, in turn, were similar to that of DNA. All curves showed a break at *ca.* 0.6 equivalent of Mg^{++} per polymer phosphate but gave a true end-point of 1.0 equivalent per P. Thus the data contradict the conclusion of Zubay & Doty (131) regarding the stoichiometry and invalidate the suggestion of Zubay (132) that the binding occurs specifically at the adenine and guanine residues. Conductiometric titrations are of necessity performed on solutions of DNA in water, and there is thus some question as to the native state of the polymer. For this reason and because the conductimetric titration results of Zubay & Doty (131) were in direct conflict with the earlier results of Shack and co-workers (135), the problem was reinvestigated (136). Using a titrimetric procedure, in which unbound Mg^{++} is determined directly with Eriochrome Black T, Shack & Bynum (136) find that, at pH 10.2 and low concentrations of sodium chloride (0.002 *M*), Mg^{++} is very tightly bound by both undenatured and denatured DNA, with binding by the undenatured form being more firm. This is in agreement with the earlier results (135) obtained with the less rigorous indirect and conductimetric method. Shack & Bynum (136) find the binding of magnesium ions greatly reduced by increas-

ing sodium chloride concentration and suggest that sodium at high concentration is capable of replacing Mg^{++} on all binding sites. The replacement by sodium chloride and the stronger binding of Mg^{++} to the undenatured DNA were considered good evidence for binding at the charged phosphates rather than at the amino or enolic groups of the bases.

Of possible relationship to this question is the response of DNA to changes in dielectric constant of the medium. Geiduschek (137) has shown that slow exchange of methanol for water in the solvent induces a configurational transformation in the DNA when the methanol concentration reaches 70 per cent by volume. The dramatic increase in sedimentation rate and fall in viscosity were interpreted as being caused by a collapse of the helical structure. However, since no hyperchromicity was observed, the collapse apparently occurred without disruption of the hydrogen-bonded structure. If true, this would indicate that factors other than the specific base pairing are contributing to the rigidity of the DNA molecule in solution. Rice & Geiduschek (138) have considered this problem and speculated that a hydrated counterion between two charged phosphates might provide greater stabilization for the helical structure than the interbase hydrogen bonds. Addition of alcohol might then destroy the helical structure by disrupting the crystal lattice of the shell of hydration. Alternatively, "hydrophobic bonds" between the bases might provide the major stabilizing force. Another consequence of the placing of DNA in solutions of low dielectric constant is the acquisition, by the polymer, of the property of dialyzability. Sinsheimer (139) has observed this phenomenon when the single-stranded DNA of the virus ϕX-174 was dissolved in aqueous dimethylformamide and dialyzed. Duggan (140) has observed similar behavior for the two-stranded DNA of chicken erythrocyte and calf thymus. While further study is obviously required for full understanding of this phenomenon, it seems certain that these macromolecules must have zero net charge under these conditions. A recent report of Inman & Jordan (141) on the electrophoretic behavior of DNA in partially non-aqueous medium is in accord with this view.

In the author's laboratory, it has been found that the standard procedure for precipitating DNA from aqueous salt solution, namely, addition of ethanol to 50 to 70 per cent, leads to some fractionation of the polymer. Thus slightly denatured preparations were less reactive to formaldehyde (142) and less readily hydrolyzed by micrococcal nuclease (56) after precipitation and redissolution. This may be an indication, albeit crude, that also under these conditions native DNA binds cations more strongly than denatured DNA. The loss of net charge could result in collapse or aggregation, or both, of the large molecules. It has also been observed that DNA that has been precipitated by alcohol from a solution buffered at neutrality will, on dissolving in the same buffer, slightly lower the pH. This would indicate that hydrogen ions as well as sodium ions may be bound to the nucleic acid.

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BIOCHEMISTRY OF VIRUSES[1,2]

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The period under review has been marked by an increasing number of biochemical studies of viruses since they promise information concerning the structure and synthesis of proteins and nucleic acids, the nature of genetic controls, the nature of mutations, and the structure of macromolecules. As in the previous review of Schramm (1), those investigations concerned with the structure and function of viral components will be treated in detail. It has not been possible to include accounts of the developments in viral genetics, serology, radiobiology, lysogeny, transduction, and chemotherapy. Discussions of these topics can be found in the transactions of various symposia (2 to 4) and in *The Viruses*, edited by Burnet & Stanley (5).

Nature of Viruses

Attempts at definition of viruses are still unsatisfactory, and it is unprofitable to consider them at length. Lwoff (6, 7) has contended that a virus is not to be confused with either organisms, molecules, cell organelles, or infectious agents; a virus is:

> an entity with an organized infectious phase, containing proteins, possessing one type of nucleic acid, reproduced from genetic material, and unable to grow and to undergo binary fission and devoid of a Lipmann system (i.e., an energy generating and capturing system).

Burnet (8, 9) doubts that all of the agents included by such a definition necessarily possess a common biological relationship. He considers animal viruses as replicating agents or microorganisms with a number of special properties and realizes that his definition includes both the rickettsiae and the psittacosis group. Members of the psittacosis group would not be included under Lwoff's definition, since they contain both RNA and DNA and appear to possess intrinsic synthetic abilities. One point emphasized by Burnet (9) is that all viruses show an eclipse phase. In this connection, Litwin (10) has recently shown that psittacosis does not have a typical eclipse phase and probably reproduces by binary fission.

Infectious Ribonucleic Acids

The discovery that preparations of RNA from TMV are infectious has been rapidly extended to include other plant and animal viruses. The simple

[1] The survey of the literature pertaining to this review was concluded September 1, 1959.

[2] The following abbreviations are used: ATP for adenosine triphosphate; DNA for deoxyribonucleic acid; RNA for ribonucleic acid; TMV for tobacco mosaic virus.

procedures used to obtain infectious RNA from TMV can be applied to other systems and appear to afford opportunities for studying both the nature of the nucleic acids and their effect on the host cell. The work up to 1958 on plant and animal virus systems has been reviewed by Colter (11).

Precise information as to the chemical nature of the infectious material is of paramount importance. There seems little doubt that RNA is at least the major, if not the only, component and that contamination by small amounts of intact virus can be excluded. In almost every case, one or more of the following criteria have been employed to demonstrate this point: (*a*) RNAse abolishes the infectivity of the RNA preparation without affecting (with a few exceptions) the infectivity of the intact virus; (*b*) the infectious RNA sediments at a slower rate than the intact virus; (*c*) the infectivity of the RNA preparation is not reduced by antiserum against the whole virus; and (*d*) the infectious RNA has quite different stability features than the whole virus: in some cases the preparations are much more labile than those of the intact virus and cannot be stored even in the cold, while in other instances the RNA preparations withstand temperatures which normally inactivate the intact virus. The infectious material is usually reported to be precipitated by ethanol and by 1 *M* NaCl, which are properties expected for RNA. However, none of the preparations is chemically pure, and the presence of minor protein or peptide components is usually reported. While some progress has been made in determining the molecular weight of a number of infectious preparations, exact data are lacking in most cases.

It should be emphasized that no reports of infectious preparations containing DNA as the major component have been made. In part, this may be attributed to the fact that DNA from such animal viruses as the pox or adenoviruses have not been tested. Work with partially degraded bacteriophage, which was originally interpreted as a possible example of an infectious DNA, now shows that the infectious material contains protein as well as DNA, both of which are essential for infectivity (12, 13). However, it also seems possible that important structural differences between RNA and DNA may be involved in their possible role as infectious agents. Present evidence indicates that the molecular weight of the DNA from a variety of sources is 7×10^6 or higher (14), while the molecular weight of RNA is of the order of 2×10^6 (see below). This difference in size, as well as the fact that DNA in every case studied [except that of ϕX-174 (15)] appears to constitute a double-stranded helix, in contrast to the single-stranded structure of viral RNA, may prevent the easy introduction of DNA into a susceptible host cell. It would be of great interest to ascertain the infectivity of the single-stranded DNA from ϕX-174 and of the DNA from various animal viruses.

Properties of infectious RNA from TMV.—In addition to the older methods, which used agitation with water-saturated phenol or 1 per cent dodecyl sulfate to remove the viral protein, infectious RNA has now also been obtained from TMV by heat denaturation of the viral protein at 90° for 1 to 3

min. (16, 17). It is relevant that all the known methods for preparing RNA from TMV do yield infectious material.

In a series of papers, Gierer (18, 19) has amplified his earlier claim that the infectious RNA corresponds to the intact RNA core of the virus. The electron micrographs obtained by Hart (20) show the existence of one large molecule of RNA in the virus particle, but, when prepared by the phenol method, TMV RNA is partially degraded and is not homogeneous in the ultracentrifuge. A molecular weight of 1.7×10^6 was determined by Gierer, who used viscosity and ultracentrifugal measurements, and appropriate corrections in view of the degradation products were made to yield a value of 2.1×10^6. This value agrees with the 5.1 per cent nucleic acid content of TMV recently obtained by Knight & Woody (21). Based on the molecular weight of 39×10^6 for TMV (17), this gives a value of 2.0×10^6 for the molecular weight of the RNA. The conclusions of Gierer have been confirmed and supported by experiments in several laboratories. Loring, Fujimoto & Eng (22) regard their ultracentrifugal data as support of Gierer's dimensions, and Cheo, Friesen & Sinsheimer (23) report that the infectivity is always in the leading or heaviest fraction in the ultracentrifuge. Boedtker (17) found that RNA prepared by heat denaturation yielded material that was homogenous and gave a relatively narrow distribution in the ultracentrifuge with S_{20} values from 27 to 30. Light-scattering, sedimentation, and viscosity measurements gave a molecular weight of $1.9\pm0.16\times10^6$. Commoner (16, 24) believes that a loss of as little as 2 per cent of the nucleotides from the single RNA chain of TMV abolishes infectivity.

Objections to these conclusions have been raised by Fraenkel-Conrat and his collaborators (25, 26), who claim that there is still a possibility that the infectious RNA may be an aggregate of smaller polynucleotide subunits. This is supported by the observation that aggregation occurs in the presence of the salt used in the physical studies and that the addition of yeast RNA retards or prevents the reconstitution of the intact virus from protein subunits and viral RNA. Loring and co-workers (22, 27, 28) have suggested that the small amount of iron found in TMV (10 to 11 atoms per mole of TMV) might hold such subunits together, but Haschemeyer, Singer & Fraenkel-Conrat (29) regard these traces of metal as contaminants in the phenol used to prepare the RNA.

Gierer (19) has concluded from a study of the kinetics of RNAse degradation that the RNA from TMV is a single-stranded molecule. The rapid decrease in viscosity observed is interpreted as being caused by breaks in the single-chain of the RNA. This view is supported by the study of the kinetics of heat inactivation of the RNA of TMV by Ginoza (30, 31). The loss of biological activity of infectious RNA occurs with moderate heat evolution ($\Delta H = 19$ kcal. per mole) and a negative entropy of activation (-19.5 e.u.) at neutral pH. Inactivation is interpreted as the consequence of a single hydrolytic break in the phosphodiester backbone of the polynucleotide

chain. Ginoza points out that it is likely that the bases in RNA are on the outside of the helix, not the inside as in DNA, since RNA reacts with formaldehyde and is absorbed by activated charcoal. He proposes a model of a single-strand RNA as a helix of 40 A radius in the TMV particle. The 50 bases per turn of the helix are aligned parallel to the longitudinal axis of the virus particle. A central hole, 20 A in diameter, extends the length of the RNA helix. The total length of the helix would be 33,000 A. For a molecular weight of 2×10^6, this requires a spacing between adjacent P atoms on the coil of 5 A. Data from the x-ray diffraction experiments of Franklin & Holmes (32) support such an interpretation and indicate that the RNA exists as a single molecular chain with a spacing of 5 A for the adjacent phosphorus atoms.

While it appears that RNA does not have a secondary structure involving hydrogen bonding in the TMV rods, there is evidence that the RNA of TMV can form stable configurations involving secondary bonding in solution. Doty *et al.* (33) believe that RNA contains small helical regions involving approximately half of its nucleotides. Haschemeyer, Singer & Fraenkel-Conrat (29) also believe that RNA from TMV can be transformed from a random coil to a more orderly configuration under the influence of moderate concentrations of divalent cations or of high concentrations of monovalent cations, as indicated by changes in its absorption at 258 mμ, its optical rotation, and its sedimentation properties. Both forms are interconvertible and equally infectious.

The important question of the presence and possible role of viral protein in the preparations of infectious RNA still remains unanswered. Although the amount of material present is about 0.1 to 0.5 per cent of the RNA by weight, there is the possibility that some peptide-like material is uniquely associated with active RNA and plays a functional role (27, 34). Takahashi *et al.* (35) have found that only a small portion of the RNA extracted from TMV is infectious, and that this fraction chromatographs differently from the bulk of the material. Further, the infectious fraction was found to contain detectable protein, although it cannot be determined whether this is a contaminant or a necessary component of the infectious material. Pirie (36) has presented evidence that the infectious RNA contains a component susceptible to oxidation and accounting for the marked instability of infectious RNA preparations. However, Boedtker (17), as well as Fraenkel-Conrat & Singer (37), ascribe the instability of the infectious preparations to the invariable presence of leaf RNAse.

Studies by Schuster & Schramm (38) and Gierer & Mundry (39), of the action of 1 *M* nitrous acid at pH 4.1 to 4.3 on RNA isolated from TMV have unexpectedly revealed that mutants of TMV are produced by this treatment as a result of the conversion of part of the amino groups of the purine and pyrimidine bases to the corresponding hydroxy compounds. Because of the large number of TMV particles required to produce a lesion, Bawden (40) is still of the opinion that the possibility of selection by the above treat-

ment must be considered. However, Vielmetter & Wieder (41) have clearly shown that treatment of bacteriophage T2 with HNO_2 can produce plaque type mutants: under conditions in which only one virus of 10,000 survived, 3 per cent of the surviving particles gave rise to mutants, some of which were stable while others reverted. Although bacteriophage T2 contains DNA instead of RNA, these results support the claim that TMV mutants can be produced.

The difficult problem of nucleotide sequence in the RNA of TMV has been attacked by Reddi (42 to 44) and Hart (45). Study of partial enzymatic digests indicated that the arrangement of the nucleotides differs from that expected on a random basis. Reddi has also shown that RNA from different strains of TMV has different nucleotide distributions.

TABLE I

INFECTIOUS RIBONUCLEIC ACIDS FOR PLANTS

Source of RNA	Extraction Method	RNAse Sensitive	Relative Infectivity*	Reference
Purified tobacco ringspot virus	Heat denaturation	Yes	0.1–1%	Kaper & Steere (46)
Purified turnip yellow mosiac virus	Heat denaturation	Yes	0.1–0.5%	Kaper & Steere (47)
Tomato bushy stunt virus	Detergent and phenol	Yes	0.1%	Rushizky & Knight (48)
Tobacco leaves infected with cucumber mosiac virus I	Phenol	?	*ca.* 5%	Welkie (49)
Cucumber leaves infected with necrotic ringspot virus	Phenol	?	*ca.* 1%	Diener & Weaver (50)

* Relative infectivity is the ratio of the amount of RNA in an intact virus preparation to the isolated RNA that gives the same infectivity.

Infectious nucleic acids for other plants.—Successful attempts have been made to isolate preparations containing infectious RNA from other plant virus systems (see Table I), although these have been confined, as yet, to the small spherical plant viruses. The infectivity of the RNA (as compared to the intact virus) obtained from these spherical viruses by three different procedures was similar to that found for RNA isolated from TMV. Identification of the isolated material as RNA has rested for most cases on loss of infectivity on treatment with RNAse, except in the case of the necrotic ringspot virus where RNAse prevents infection by the intact virus (51). In every case, these preparations contained varying amounts of protein (0.3 per cent or more), the significance of which has not been determined. Measurements of molecular size and other characteristics have not been reported, although possible differences between the RNA from the spherical plant viruses and that from the rod-like viruses, such as TMV, would be of interest.

Infectious ribonucleic acids for animal tissue.—The phenol method has

TABLE II

INFECTIOUS RIBONUCLEIC ACIDS FOR ANIMAL TISSUES

Viral Type and Source of RNA	Relative Infectivity*	Molecular Weight	Reference
Myxoviruses			
Chorioallantoic membrane infected with influenza Type A	0.4%	?	Maassab (52)
Partially purified influenza virus A	?	?	Portocala *et al.* (53)
Arbor viruses			
Ehrlich ascites cells infected with Mengo encephalities virus	0.1%	$1-2\times10^6$	Colter *et al.* (54, 55)
Ehrlich ascites cells infected with West Nile encephalitis virus	0.1%	$1-2\times10^6$	Colter *et al.* (56)
Mouse brain infected with Semliki Forest encephalitis virus	0.1%	?	Cheng (57)
Virus-free supernatant from ascites cells infected with encephalomyocarditis virus	?	?	Huppert & Sanders (58)
Mouse brain infected with Murray Valley encephalitis virus	1%	?	Ada & Anderson (59)
Mouse brain or chick embryo infected with encephalitis virus	?	2×10^6	Wecker & Schafer (61); Wecker (60)
Western equine encephalitis virus	.0001%	?	Wecker (62)
Mouse brain infected with encephalomyelitis virus and partially purified virus	?	?	Franklin, Wecker & Henry (63)
Pig kidney cultures infected with foot-and-mouth virus and partially purified virus	?	?	Brown *et al.* (64, 65)
Mice infected with foot-and-mouth virus	?	3.1×10^6	Mussgay, Strohmaier (66 to 68)
Cattle tongues infected with foot-and-mouth virus	?	?	Spuhler (69)
Enteroviruses			
Brain and spinal cord of hamsters infected with polio virus Type II	.1%	$1-2\times10^6$	Colter *et al.* (56)
Partially purified polio virus Type I and II	?	?	Alexander *et al.* (70, 71)
Highly purified polio virus Highly purified Coxsackie virus	?	"High"	Schaffer & Mattern (72)
Partially purified polio I Partially purified Coxsackie A-1 Partially purified Coxsackie B-1 Partially purified Echo 8	?	?	Holland *et al.* (73, 74)

* See Table I.

also permitted the isolation of infectious RNA from various infected animal tissues and from purified animal viruses. The list in Table II represents an impressive number of infectious agents but only a fraction of those yet to be investigated. For convenience, the reports are grouped under the viral type agreed upon at the Sixth International Congress of Microbiology, Rome, Italy, 1953. It seems likely that the relationships implied between two viruses listed under the same type, while useful, may have to be reconsidered when the properties of infectious preparations are compared. It should be noted

that no infectious nucleic acid has been reported from either pox or adenoviruses, which contain DNA. Various methods of assay have been used to determine infectivity, including inoculation into various sites of whole animals, into chorioallantoic membranes of eggs, and into tissue cultures. The relative infectivities of the isolated RNA preparations, when compared to those of the intact viruses, are similar to those found with plant viruses. However, the standard phenol procedure has not yielded infectious preparations from several purified viruses, including three arbor viruses, Bunyamwera encephalitis virus (56), encephalomyocarditis virus (58), and western equine encephalitis virus (62). Both Ada *et al.* (75) and Maassab (52) have failed to obtain infectious RNA from purified influenza. Portocala and co-workers (53), on the other hand, claim to have obtained infectious RNA from influenza; they have made the new and possibly important observation that the filamentous form of influenza, characteristic of new influenza infections, is produced after infection with RNA from a non-filamentous spherical form.

In spite of his failure with purified virus, Maassab (52) was able to extract infectious RNA from chorioallantoic membranes infected with influenza. Similarly, Huppert & Sanders (58) have obtained infectious RNA from virus-free extracts of ascites cells that had been infected with encephalomyocarditis virus but not from the virus itself. These apparent contradictions may be explained by the experiments of Wecker (62), who found that warm phenol (*ca.* 50°) would extract infectious RNA from western equine encephalitis virus, while cold phenol, though without effect on the intact virus, did yield infectious RNA from infected cells. It appears that a number of viruses can be present intracellularly as protein-free RNA, readily extractible by cold phenol, but once the RNA is enclosed in a protein it is resistant to extraction. For example, the amount of infectious RNA that can be extracted from Murray Valley encephalitis virus by cold phenol is markedly increased by heating. One would expect that infectious nucleic acid could be obtained from almost any RNA virus if the proper extraction procedure could be devised. The particular features of the virus protein structure that determine the ease with which RNA can be extracted are not understood; the presence of lipids in the myxoviruses seems to inhibit extraction by cold phenol.

Conviction that RNA is the essential component of phenol extracts of viruses rests largely on the observation that the infectivity is destroyed by RNAse. Additional support to this conclusion is afforded by other properties of the extract, such as insolubility in ethanol and 1 *M* NaCl, heat stability differing from that of the intact virus, and infectivity unaffected by antisera to the intact virus. In general, phenol preparations contain components other than RNA. Habermann (76) reports the presence of 0.2 to 1.5 per cent peptides in RNA from yeast, ascites cells, mouse liver, and kidney cells. The peptides are characterized by large quantities of dicarboxylic amino acids tightly bound to the RNA, which cannot be removed by the usual purification procedures.

Franklin & Wecker (77) have found that 1 *M* hydroxylamine at pH 7.0

inactivates the RNA of mouse encephalomyelitis virus and suggest, mostly on indirect evidence, that hydroxylamine reacts with the RNA itself or some component closely associated with the RNA. They propose that the site of attack is an amino acylester formed between an amino acid and the RNA. If it is shown that such a bond is required for the infectivity of the material, this would argue against RNA alone as the infectious material.

Although the data are not extensive, it appears that the infectious materials in the phenol extract have molecular weights corresponding to the total RNA of the intact virus particle. Values obtained in the ultracentrifuge and by light-scattering are of the order of $1–3\times10^6$ (see Table II), which is similar to that for TMV RNA. Both Gierer (19) and Timasheff *et al.* (78) have examined RNA from preparations that were obtained by the phenol method from various animal tissues, and they agree that the major component has a molecular weight of $1.2–1.8\times10^6$. The observation of Brown *et al.* (55) that the infectious material from encephalitis virus could be purified by the use of a column of methylated bovine serum albumin seems promising.

Holland, McLaren, & Syverton (73, 74) have had interesting results from experiments with infectious RNA obtained from Type 1 polio, Coxsackie A-9, Coxsackie B-1, and Echo viruses. These preparations are able to infect non-primate cells from rabbits, swine, mouse, guinea pig, chicken, and hamster, which are not attacked by the intact virus. The virus produced is identical to that from which the RNA had been obtained. These cells lack receptors capable of interacting with the intact virus, although the viral RNA, as such, is able to penetrate and reproduce within the host cell. These studies have important implications with reference to the relation of infectious agents to immunity and the nature of virus-induced tumors.

Plant Viruses

TMV.—Since the chemistry of the RNA from TMV has already been discussed, this section will deal with the protein structure of the virus and the reaction involved in initiating infection. Independently, Anderer (79) and Ansevin & Lauffer (80) have prepared from TMV protein components having a molecular weight of about 18,000. Ansevin & Lauffer found that, after the usual degradative treatment at pH 10.3, extreme dilution was necessary to obtain unaggregated subunits. When concentrated, this material readily aggregated with RNA of TMV to form infectious rods. This size for the subunits is in agreement with end-group and x-ray analysis for the protein subunits, but previous attempts at isolation had given material with molecular weights of the order of 90,000.

The first amino acid analysis of RNA-free TMV protein has been reported by Ramachandran (81). The results, in general, agree with values obtained in analyses of the whole virus and show that there are 164 amino acid residues corresponding to a molecular weight of 18,270 per minimal peptide chain or subunit. The sequence of amino acids after trypsin digestion in six peptides containing 33 of the 164 amino acids has been determined by Gish, Rama-

chandran & Stanley (82, 83). Ramachandran & Narita (84) have also found that 12 of the 19 aspartic acid residues and 9 of the 17 glutamic acid residues exist as the corresponding amides in the peptide chain.

Studies on the N-terminal sequence of the TMV protein by Narita (85, 86) have shown that the N-terminal amino group is acetylated. Acetyl-seryl-tyrosine has been isolated from chymotrypsin digests of five strains of TMV and identified by comparison with synthetic N-acetyl-seryl-tyrosine. This is the first report of the existence of an acetyl peptide in nature. Anderer (87) has confirmed Narita's findings and has also shown that aspartyl-proline peptides are susceptible to mild acid treatment. This explains the earlier incorrect suggestion that the appearance of proline after mild acid treatment is evidence for the location of proline at the N-terminal end of the peptide chain. Narita (88) has examined the N-terminal structure of cucumber virus 4, a virus which on biological evidence is thought to be related to TMV, and found that it is also acetylated but has the sequence N-acetyl-alanyl-tyrosine.

The bonds linking the protein subunits to form the virus rods have not been specifically identified, but Fraenkel-Conrat (89, 90) has concluded that no covalent bonds are involved and that undissociated carboxyl groups probably participate by hydrogen bonding. The experiments of Koshland, Simmons & Watson (91) support this view. They have shown that the decrease in pH observed upon denaturation of TMV originated presumably from the unmasking of carboxyl groups rather than from the cleavage of phosphotriester bonds. Kramer & Gunter (92) have produced evidence that the basic groups of the reaggregated subunits are situated inside the rods, and it seems possible that these basic groups form hydrogen bonds with the hidden carbonyl groups of the RNA. More than one type of bond appears to be involved in the formation of the virus structure, and Fraenkel-Conrat (90) has shown that the TMV protein contains one masked sulfhydryl group per subunit capable of giving stability to the structure by secondary bonding. Lauffer *et al.* (93) have shown that the polymerization of the subunits is temperature-dependent and is favored by higher temperatures. They believe that the water of hydration decreases during polymerization so that increasing the temperature favors the formation of the less hydrated polymer.

Wildman (94) has reviewed the earlier information on the process of infection by TMV. Benda (95, 96) has shown that the direct mechanical inoculation of virus particles into single cells greatly increases the efficiency of infection and that the appearance of lesions, even under these conditions, can be inhibited by RNAse. All workers in this field agree that the release of the RNA from its protein cover is one of the early stages in the infectious process. The time required for this reaction is calculated from the difference in the time required for the appearance of new virus after infection with whole virus as compared with the time required for growth after infection with RNA. Siegal, Ginoza & Wildman (97) and Kassanis (98) found that 2.5 to 5 hr. was the time required to release the RNA from its covering;

Fraenkel-Conrat *et al.* (99) reported 5 to 10 hr.; and Schramm & Engler (100) reported 10 to 12 hr. These differences appear to reflect the different techniques used, and it is not clear whether all values measure the same reaction since the release of RNA may involve several steps. Engler & Schramm (101) have also shown that a considerable amount of infectious RNA is produced before the synthesis of the intact virus can be detected.

Bushy stunt virus.—The size of the protein subunits in this small spherical virus has been studied by Hersh & Schachman (102). The virus was degraded with sodium dodecyl sulfate under neutral conditions, and the smallest protein components were shown to have a molecular weight of 6×10^4, i.e., half that of the crystallographic unit and double that calculated from the amino acid composition. The virus contains about 120 such units and appears to conform to the symmetrical requirements usually associated with the small spherical viruses. Niu, Shore & Knight (103) have reported that bushy stunt, like TMV and cucumber 4 virus, does not have a free N-terminal group.

Animal Viruses

Myxoviruses.—The myxoviruses, such as influenza, have a more complex structure than some of the other animal viruses, such as entero- and adenoviruses. Frisch-Niggemeyer (104) has proposed a model for the structure of influenza virus that is based on thin section electron micrographs and physical, chemical, and serological investigations. The virus particle is spherical and has a diameter of 100 mμ and a particle weight of about 300×10^6. There is a central electron dense core, 54 to 60 mμ in diameter in the virus particle, which is believed to contain the whole of the viral RNA and to be composed of 70 ribonucleoprotein particles, each 12 mμ in diameter. These particles constitute the complement-fixing antigen (CFA or g-antigen). Kroeger & Kempf (105), by measurements of the susceptibility to radiation damage, find that the structure responsible for infectivity has a diameter of 48 mμ and presumably corresponds to the central core. Frisch-Niggemeyer suggests that the core contains about 20 per cent of the total protein, and Hoyle & Finter (106) find that the complement-fixing antigen (presumably core protein) contains about 15 per cent of the methionine of the virus particle. Surrounding this core is a protein shell (molecular weight 1.6×10^6), which is responsible for interference with infection by other viruses (see later section). A lipid coat, about 8 mμ thick, surrounds this inner protein layer and is believed to constitute the region of low electron density seen in thin sections and to explain the ether sensitivity of the virus. In Frisch-Niggemeyer's model the HA-particles, which possess the hemagglutinating activity of the virus, are embedded in this lipid layer. There are 70 HA particles, each about 12 mμ in diameter and containing 20 per cent of the viral protein; he pictures the number and size of the HA particles as being similar to the ribonucleoprotein particles in the core. Kroeger & Kempf report that the HA particles have a much larger diameter than this, from 17 to 23 mμ, and Hoyle &

Finter find only 15 per cent of the virus methionine in the HA particles. The size and number of these particles is thus not certain. Finally, there appears to be a layer of glycoprotein or polysaccharide on the outside of the virus particle.

The fact that small ribonucleoprotein particles can be isolated from ether-treated influenza virus has been known for some time, and Frisch-Niggemeyer depicts the core of influenza virus as though it were composed of tightly packed identical spherical particles. There are reasons to believe that this cannot be the correct arrangement. The radiation data indicate that the infectivity is a property of the whole central structure, which includes all the ribonucleoprotein. This is a strong argument against the possibility that each of the small particles might be infectious. Further, it should be noted that, whereas all infectious RNA preparations from plant and animal viruses have a molecular weight of the order of 2×10^6, the small particles contain RNA corresponding to only about 3×10^4 each. The total RNA content of the influenza particle (.8 per cent of 300×10^6), however, corresponds to 2.4×10^6. In this connection it may be pointed out that the so-called incomplete forms of influenza virus have both a low infectivity and a lower RNA content. The organization of the RNA in the central core of influenza virus is not understood, and the reason for its lability to ether treatment is uncertain.

The possible presence of host materials in influenza virus preparations greatly complicated earlier interpretations of analytical results, but current purification procedures seem to yield virus free of extraneous host material. There is now considerable evidence pointing to the presence of host material as an integral part of the virus. Frommhagen, Freeman & Knight (107, 108) have analyzed both highly purified virus preparations and chick allantoic membranes and fluids for their lipid and polysaccharide constituents. They report that the relative amounts of cephalin, sphingomyelin, lecithin, and unesterified cholesterol are the same in both purified virus and host material. Similarly, the relative amounts of galactose, mannose, fucose, and amino sugars are the same in both preparations. It is generally believed that influenza virus is assembled from its component parts near the cell membrane and that host cell materials derived from the cell membrane constitute the coat of the virus particle. This view has been elaborated by Franklin (109), who has concluded that there is a correlation between the lipid content of animal viruses, their ether sensitivity, and the mechanism of their release from the host cell. Viruses such as influenza (34 per cent lipid) and western equine encephalitis (40 per cent lipid) are ether sensitive and appear to be released by a continuous process from the host cell, whereas viruses such as poliomyelitis and adenoviruses contain no lipid, are not ether sensitive, and appear to be released by a burst-like process. Isotope data show that the host cell-like viral constituents in influenza virus are synthesized before infection and suggest that they are incorporated in a relatively intact form into the virus. Franklin proposes that the final step in viral maturation or com-

pletion consists of obtaining "a protective lipid coat from lipid structures at the cell periphery as the particles pass to the external cell surface." This hypothesis could be tested by comparing the nature of the lipid and polysaccharide components of virus grown in different types of host cells. These views about the origin of what must be considered essential viral components imply that these materials are synthesized and added as a coating to the virus particle in a manner that is only distantly controlled by the virus genome. This conclusion is in sharp contrast with those generally held by workers in other systems.

The process by which influenza RNA enters the susceptible cell has been partially elucidated. Hoyle & Finter (106) infected chick chorioallantoic membrane with influenza virus containing S^{35} methionine. After $1\frac{1}{2}$ hr., 10 to 20 per cent of the S^{35} appeared in the free amino acid fraction while the bulk of the S^{35}-containing protein remained as virus protein coat, presumably on the outside of the cell. They suggest that during invasion the viral coat is opened and that only the ribonucleoprotein portion passes into the cell, where its protein is partially hydrolyzed. The virus protein envelope and the hemagglutinins apparently remain on the cell surface. LeClerc (110) has shown that influenza infection is inhibited by the addition of RNAse within 1 to 2 hr. after infection, but not later or earlier. This has been confirmed by Burnet, Lind & Perry (111). It is not known whether the RNAse attacks the viral RNA after release from its covering or whether it affects other processes involving RNA. Klamerth (112) has studied P^{32} incorporation in various components of infected cells and suggests that a more labile RNA different from that originally in the cell, is formed during the early stage of infections.

The role of the viral enzyme neuraminidase in influenza invasion is still not clear. Gottschalk (113, 114) has shown that the influenza neuraminidase splits the ketosidic bond in 6-α-D-sialyl-N-acetyl galactosamine, and he suggests that host cell receptors contain this type of structure. However, Burnet & Lind (115), in a study of the effect of lowered temperatures on the first stage of infection, conclude that the initial union does not aid infection and doubt whether enzyme action is necessary for successful infection.

Burke, Issacs & Lindeman (116 to 118) have reported that heat and ultraviolet-inactivated influenza virus initiate the production of a factor, called the interferon, in chick chorioallantoic membranes. The addition of partially purified interferon to fresh membranes prevents infection by added virus. Interferon cannot be isolated from the virus particle but is believed to be a protein synthesized in the host cell after infection. The mechanism of its action is not clear, but its appearance and properties have been confirmed by Tyrrell (119) and Henle *et al.* (120).

The properties of the hemolysin of mumps virus have been studied by Moberly *et al.* (121) and Soule *et al.* (122). The hemolysin appears to split sphingomyelin from the stromal lipoprotein. Sixty per cent of the phosphatide of the purified virus is sphingomyelin, and the ability to release sphingo-

myelin is thought to be involved in the mechanisms by which the virus matures and is released from its host cell.

Enteroviruses.—Crystalline poliovirus has been subjected to x-ray diffraction study by Finch & Klug (123). They have concluded that the virus particles consist of a nucleic acid core surrounded by a protein coat of 60 small protein molecules. The structure is similar to that of the small plant viruses and seems to indicate that a requirement for spatial symmetry may be responsible for this type of structure in all small spherical viruses. Cross-section measurement by radiation (124) has indicated a diameter for the poliovirus of 24 to 32 mμ, which is in agreement with electron microscope values of 27 ± 2 mμ. Similar sizes were obtained for the related Echo 1 and Echo 7 viruses by cross-section measurements. The early stages of polio infection have been shown to include specific adsorption to a lipoprotein receptor (125, 126) followed by an eclipse period (126, 127), in which the nucleic acid is released from its protein coat. Ackermann, Payne & Kartz (128) have claimed that a toxin that is produced by polio infected HeLa cells is responsible for the cytopathic effects usually observed.

Hoof-and-mouth disease virus has been purified by Bachrach & Breese (129), and the diameter was shown to be 22 mμ in the electron microscope. Strohmaier & Mussgay (130) have calculated that the particle weight of this virus is 5×10^6 and that its RNA has a molecular weight of about 3.1×10^6. If confirmed, this would be the highest concentration of ribonucleic acid found in any virus particle.

DNA viruses.—The structure of an adenovirus has been clearly revealed by electron micrographs of Horne *et al.* (131), who used phosphotungstic acid as a stain. This virus has a diameter of 70 mμ and an icosahedral form in which the DNA is surrounded by a shell of 252 subunits, which have a diameter of 7 mμ each. Herpes virus shows a similar structure, although the subunits appear to be hollow and ring-shaped. The changes occurring in HeLa cells after infection with herpes virus (132 to 135) resemble, in general, the changes which take place in bacteriophage systems: the herpes virus attaches, the cell nucleus is broken down, and the virus disappears. Infection initiates an increased production of DNA with little or no change in RNA. It is possible that these events represent a basic sequence for all DNA viruses.

Tumor viruses.—Over twenty reviews on this subject have appeared in the last two years [see Anderson & Law (136)]. The structure of Rous sarcoma virus has been studied in thin sections by Haguenau *et al.* (137) and in purified preparations by Epstein & Hold (138) and Bather (139). Intracellular particles have been definitely identified as the infective agent. The particles are slightly ovoid (75 to 80 mμ in diameter) and have an outer and inner membrane and a central electron dense core that contains the viral RNA and is 1.4 per cent of the weight of the particle. Forty-seven per cent of the virus is lipid material, of which about one-half is phospholipid. The evidence that DNA is absent is conclusive. An attempt to obtain infectious RNA from Rous virus by the phenol method was unsuccessful (140).

Schmidt (141) has found that the Rous virus can be inactivated reversibly by *p*-chloromercuribenzoate, and he believes that sulfhydryl groups may be involved in viral attachment. Temin & Rubin (142) have shown that Rous virus is released from chick embryo cells in tissue culture at a uniform rate and that isolated cells in microdrops continue to divide after release of virus particles. This observation of cell division occurring after viral release is unique among animal viruses. Bernhard *et al.* (143) and Bonar & Beard (144) have continued their chemical studies of the avian leukemia viruses. The viruses that cause avian myeloblastosis and erythroblastosis are similar to the Rous sarcoma; they have a diameter *ca.* 80 mμ, an electron dense core, and a limiting outer membrane. The myeloblastosis virus is free of DNA and contains 2.2 per cent RNA and a large amount of lipid.

Recent measurements by Moore *et al.* (145) of the size of the rabbit papilloma virus from thin sections reveal that this DNA virus is smaller and more symmetrical than previously described. The virus has a diameter of 33 mμ and appears spherical.

Some of the physical properties of mouse milk tumor virus have also been determined (146, 147). Two active agents have been identified in filtrates of milk from RIII high-breast-cancer mice, one with a diameter of *ca.* 100 mμ, the second with a diameter of 20 to 30 mμ or less. These two agents correspond, respectively, to the whole particle seen in thin sections and to its central dense nucleoid. The particles, like influenza viruses, appear to be formed near the cell membrane. There is little chemical information about the SE mouse polyoma viruses or the mouse leukemia viruses (136). However, rapid development of biological studies promises that these tumor viruses will soon be chemically characterized.

Bacterial Viruses

A comprehensive treatment of bacteriophages by Adams (148) has appeared; several articles dealing with phage genetics, invasion, multiplication, and radiobiology can be found in Volume 2 of *The Viruses* (5); and Evans (149) has recently reviewed the chemistry of phage particles.

Constituents of bacteriophage T2.—A list of the constituents of bacteriophage T2 is given in Table III. This Table shows not only the great complexity of this virus but also that only a beginning has been made in separating and characterizing many of the constituents. The heading "molecular weight" is used for convenience in Table III and in the text, although the term "particle weight" for whole virus is more correct. The observation of two different sedimentation rates that are pH dependent has raised a question regarding the particle weight of bacteriophage T2. This question has now been resolved. Taylor, Epstein & Lauffer (150) calculated the weight of T2 from the sedimentation and diffusion constants at pH 5 and arrived at a value of 220×10^6, while measurements made at pH 7 gave a value of 181×10^6. These values eliminated aggregation as the explanation of the dual sedimentation phenomenon. Bendet *et al.* (151, 150, 171) proposed two different

TABLE III

CONSTITUENTS OF BACTERIOPHAGE T2

Constituents	Molecular Weight	Molecules per T2	Fraction (percentages)	References
Intact T2	220×10^6	1	100	Taylor *et al.* (150); Bendet *et al.* (151)
	215×10^6	1	100	Cummings (152)
	214×10^6	1	100	Calculated from (153) and (154)
Head Constituents				
(1) DNA	131×10^6		61	Jesaitis (155)
	45×10^6 / 12×10^6	1 / 6–7	58	Leventhal & Thomas (156); Thomas & Knight 157)
	$19–25\times10^6$	?		Cohen (158)
(2) Protein subunits	8.7×10^4	?	*ca.* 28	Van Vunakis *et al.* (159)
(3) Internal head Protein	?	?	3	Levine *et al.* (160)
(4) Polyamines				
Spermidine	145	*ca.* 9400	0.6	Ames *et al.* (161, 162)
Putrescine	88	*ca.* 62,000	2.5	Ames *et al.* (161, 162)
Tail Constituents				
(5) Contractile protein	*ca.* 7×10^6	1	*ca.* .3	Kozloff & Lute (163)
(6) Phage lysozyme	*ca.* 2×10^4	?	?	Koch & Dreyer (164)
(7) Tail core	*ca.* 3×10^6	1	*ca.* 1.5	Kellenberger & Arber (165)
(8) Tail plate	(1.5×10^6)	1	*ca.* 0.5	Brenner *et al.* (166); Setlow (167)
(9) Tail fibers	186,000	6	0.5	Brenner *et al.* (166); Pollard & Setlow (168)
(10) ATP	623	90 ± 7*	0.03	Kozloff & Lute (163)
(11) dATP	607	15 ± 2*	0.004	Kozloff & Lute (163)
(12) Ca^{++}	40	101 ± 15*	0.002	Kozloff & Lute (169)

* Recalculated from the original reported values that use $1.8\times10^{-11}\gamma$P per T2 instead of $2.3\times10^{-11}\gamma$P per T2.

pH-dependent orientations of the phage tail fibers as a cause of the dual sedimentation and concluded that, because of this and the possibility of orientation of the particle during sedimentation, the molecular weight could not be accurately determined by sedimentation and diffusion measurements. However, Cummings (152) has found that sedimentation of T2 varies with temperature as well as pH so that, when sedimentation and diffusion measurements on the two forms are made under proper conditions of pH and temperature, the molecular weight obtained is the same for both forms, i.e., 215×10^6 This value is similar to that given by Taylor *et al.* (150) for the determination at pH 5, whereas the value of Taylor *et al.* at pH 7 is incorrect, because partial conversion of the slower sedimenting form into the alternate form occurs at the low temperature of the diffusion bath. Cumming suggests that the two forms have different head configurations and, this explanation seems more likely than that proposed by Bendet *et al.* (151, 170,

171). Calculation of the particle weight based on the P content, light-scattering (153), and direct particle count (154) gives a value in excellent agreement with those obtained by the measurements of the sedimentation and diffusion rates.

The DNA content of the T2 particle as calculated (153, 154) from the P content of whole T2 (5.1 per cent) and from the P content of the isolated DNA (8.5 per cent) is 61 per cent of the total weight and corresponds to a molecular weight of 131×10^6. A value of 125×10^6 for the total DNA can also be calculated from the data of Thomas & Knight (157). Whereas the molecular weight of the DNA *in situ* inside T2 is not known provisional values of $19–25 \times 10^6$ for T2 DNA in solution have been reported by Cohen (158), and Barber, *et al.* (172) have reported a similar value of $15–26 \times 10^6$ for the DNA of a Salmonella phage, called D4. Levinthal & Thomas (156), and Thomas & Knight (157) have claimed that there is one large piece of DNA of molecular weight of 45×10^6 and a number of smaller pieces with molecular weights of about 12×10^6. This view of the bipartite nature of the DNA is supported by experiments of Stent *et al.* (173). However, this question, which is of the greatest theoretical and practical importance, is still unanswered. Davison (174) has shown that the rate of emptying and the size of needle used with the hypodermic syringe for filling the ultracentrifuge cell cause variations in the sedimentation rate of DNA of T2 and T4, a result, presumably, of the shearing forces involved in the passage of the DNA through the needle. Under conditions where the shearing forces are minimal, S_{20} values of 53 or more are obtained, while, under conditions where the shearing force is large, S_{20} values as low as 17 have been recorded. The higher values of S_{20} are compatible with a molecular weight of 1×10^8 and suggest the possibility that the DNA of T2 and T4 may exist as a single molecule.

In addition to the pyrimidine hydroxymethyl cytosine, 6-methylamino purine has been found in both T2 and Salmonella phage by Dunn & Smith (175) in a ratio of one mole of the unusual purine to 200 moles of adenine.

The head structure of T2 contains, in addition to the DNA, a protein coat, an internal protein, and polyamines. The protein subunits of the protein coat, obtained by alkaline-detergent treatment of T2 ghosts (159), appear to be highly asymmetric molecules, although the detergent used in their isolation makes any conclusion concerning their shape hazardous, and the reported molecular weight may represent aggregates of the smaller subunits. Levine, Barlow & Van Vunakis (160) have detected, by immunological procedures, a protein inside the phage head. Its function is unknown, but it appears to be closely associated with the viral DNA and to be injected into the host cell along with the DNA. Spermidine and putrescine were found in the head of T2 by Ames, Dubin & Rosenthal (161), and Ames (162) has shown that they neutralize a large fraction of the charges of the DNA. Fraser & Mahler (176) suggest that the polyamines may act to protect the DNA from heat inactivation.

Mahler & Fraser (12) have investigated the properties of the agent called

π, which is produced from T2 by treatment with 6*M* urea and can infect host cell protoplasts. This agent, which is sensitive to DNAse, has now been found by Mahler & Fraser (12) and Sekiguchi (13) to be also sensitive to proteases such as trypsin. It, therefore, cannot be considered an infectious nucleic acid analogous to the RNA preparations isolated from plant or animal viruses. The active agent in the preparation appears to be approximately the same size as T2 and to consist of phage particles with both damaged heads and damaged tails. A structural explanation for the fact that phage altered by treatment with 6*M* urea can infect host cells without an outer cell wall has not been advanced.

Structure of the small bacteriophages, ϕX-174, and *S13.*—Revived interest in the smallest of the known bacteriophages has led to the unexpected discovery of Sinsheimer (15) and of Tessman and his colleagues (177, 178) that the DNA of bacteriophage ϕX-174 appears to be a single-stranded structure rather than the double-stranded helix assigned to DNA in other instances. Similar conclusions seem to hold also for S13, since Zahler (179) has shown that both are of a similar size, are serologically related, and have similar genetic properties. Sinsheimer (180) has developed a procedure for purifying sufficient quantities of ϕX-174 to permit its physical and chemical characterization. Molecular weight, by light-scattering measurements, is 6.2×10^6, and at pH 9 the sedimentation constant, S_{20}, is 114. The DNA, by colorimetric analysis, was *ca.* 25 per cent, corresponding to a molecular weight for the DNA of 1.6×10^6. The molecular weight of DNA isolated by the phenol procedure was 1.7×10^6, indicating that there is one molecule of DNA per virus particle.

The experimental observations that are the basis for the conclusion that the DNA is single-stranded are: (*a*) DNA from ϕX-174 does not have a complementary nucleotide structure, adenine/thymine = .76 and guanine/cytosine = 1.29; (*b*) the DNA reacts readily with formaldehyde, indicating that the amino groups of both purines and pyrimidines are accessible and do not participate in hydrogen bonds; (*c*) the effect of temperature and salt on the ultraviolet adsorption indicates that the structure is not similar to other DNA preparations, (*d*) ϕX-174 DNA is precipitated by plumbous ions, which is characteristic of single-stranded DNA obtained by heat inactivation; (*e*) light-scattering measurements show that the DNA is highly flexible in solution: (*f*) DNAse causes a decrease in the average molecular weight of this DNA in a manner expected for single-stranded DNA; and (*g*) finally, Tessman and his colleagues (177, 178), after measuring the relative sensitivities of ϕX-174, S13, and T2 to decay of P^{32} incorporated into the virus DNA, concluded that ϕX-174 and S13 are both inactivated in a manner which indicates that a single P^{32} disintegration breaks the chain and inactivates the virus, whereas T2 inactivation requires about ten disintegrations.

The fine structure of ϕX-174 has been examined by Hall, MacLean & Tessman (181) in the electron microscope. The virus is a regular polyhedron, probably a dodecahedron, with a diameter of 243 A. These investigations

show that there is a remarkable resemblance between the small spherical viruses such as bacteriophage ϕX-174, plant viruses such as bushy stunt, or animal viruses such as polio. All have a similar polyhedral structure, and all have approximately the same amount of nucleic acid, about 2×10^6. In the bacterial virus, the nucleic acid is an unusual DNA with a single-stranded structure and therefore, in this respect, is similar to the RNA of these other viruses.

Phage adsorption and penetration.—The problems involved in the invasion of *Escherichia coli* by the T-bacteriophages have been recently reviewed by Weidel (182), Garen & Kozloff (183) and Kozloff (184). Valentine & Allison (185) used several models to study the attachment of various virus particles and concluded that absorption rates for virus particles fit closely with predictions from the theory of Brownian motion. The presence of salt is thought to be necessary to neutralize the two negatively charged surfaces as they approach each other. The role of L-tryptophan as cofactor for adsorption of bacteriophage T4 has been partially clarified. Kozloff, Lute & Henderson (186) showed that the presence of L-tryptophan increased tenfold the rate of inactivation of T4 by partial complexes of zinc or cadmium, such as $Zn(CN)_3^-$ or $Cd(CN)_3^-$. Tryptophan appears to alter the structure of the tail tip to expose the susceptible bond to the metal complexes. Fildes & Kay (187) have shown that the presence of tryptophan quite specifically makes the tail of phage 3 (a phage which attacks *Bacillus coli* 518) susceptible to alteration by gentle heating, which produces a form similar to that of T4 treated by $Cd(CN)_3^-$. These tail alterations are of interest because they appear to be the same alteration undergone by the tail when it interacts with the cell wall. Kozloff and his colleagues (186) have proposed that the chemical bond involved is most probably a thiolester formed between a sulfhydryl group of one constituent and a carboxyl of another. There is evidence that zinc that is tightly bound to some normal cell wall component may be involved in causing the alteration of the phage tail (188).

The alteration of the tail leads to the exposure of the phage tail lyzozyme (164, 189), which removes those components from the cell wall that give it rigidity (190). The products produced by action of megatherium phage on megatherium cell walls have been characterized by Salton & Milhoud (191). Brown & Kozloff (189) have shown that the tail lysozyme is normally hidden but is exposed by various treatments which alter the tail structure. Panijel & Huppert (192) have independently reported similar results. Penicillin appears to remove the same components from the cell wall as does the phage tail enzyme (190, 193). Denes & Polgar (194) have reported that host bacteria treated with penicillin can be infected by T2 phage; so it appears that penicillin does not remove the site responsible for phage adsorption, and enzyme action *per se* is not necessary for infection. This is not surprising, since the activity of the phage lysozyme does not result in the detachment of the virus from its host. It seems likely that phage receptors are located on the inner lipoprotein component of the cell wall.

Almost simultaneously with the exposure of the tail lysozyme of T2, the major protein component of the tail appears shorter and thicker. Kozloff & Lute (163, 169) and Dukes & Kozloff (195) have shown that this change involves a contractile protein that resembles mammalian actomyosin. Associated with this protein are about 90 molecules of ATP, 10 molecules of dATP, and about 100 atoms of Ca^{++} per phage particle. During invasion, the protein contracts and the nucleoside triphosphate is hydrolyzed to nucleoside diphosphate and inorganic P. The contractile protein has phosphatase activity *in vitro* (195). The role of the contractile step in the infection process has not been defined. Kozloff (184) has suggested the possibility that the contraction of the tail protein might drive the tail core through the fragile inner cell membrane of *E. coli* (196). There is no direct evidence, however, that this membrane is actually penetrated, although this is usually assumed.

The forces involved in the release of the DNA from within its head membrane and in its passage through the altered tail have been examined by Kozloff & Lute (197) in model systems. The addition of compounds containing primary amino groups favors the release of the DNA at alkaline pH values. It seems possible that changes in the configuration of the head of T2 (152) might play a role in the injection of the DNA into the host cell. The tail core runs the length of the tail (165), and according to Brenner *et al.* (166), is hollow and may function as a channel through which the DNA travels. Terada & Shibazaki (198) have published an electron micrograph study of an inactivated *Salmonella sendai* phage that has a DNA fiber extending from the tip of the tail.

During infection the host cell is killed, but the mechanism involved has not been identified. Kozloff & Lute (188) have shown that the host cell could be killed in the absence of lysozyme activity. Recently, Setlow (167) has found, by measurement of the cross section to radiation damage, that the killing component of T2 phage has a molecular weight of 1,560,000. The phage component that might have this function is the structure at the tail tip known as the tail plate (166). This phage component has not been isolated nor its function identified. Setlow's value eliminates the six tail fibers as the killing component, since their molecular weight is 186,000 each (167).

Changes in host cell metabolism after phage infection.—The pioneering work of Cohen and his colleagues on the synthetic patterns in infected bacterial cells has led other investigators to turn their attention to this problem. It has become clear that the enzymatic machinery of the host cell upon infection is radically altered so that viral components are synthesized in place of host cell components. Progress has been made both in establishing the features of this change and in the mechanism by which these changes take place.

One of the early events after infection is the disruption of the host cell nuclear apparatus. Studies with T5 by Pfefferkorn & Amos (199) and Crawford (200) have shown that this can happen before the injection of the viral DNA. The viral constituent that initiates this change is not known, but one is inclined to suspect the host-killing component (see above). Associated

with this reaction is an increase in the DNAse activity in T5 and T-even infected cells (199 to 202). The mechanism involved in the increased DNAse activity is not entirely clear, although the inhibition of this reaction by chloramphenicol supports the idea that new enzyme is formed (200) .There is also evidence in the T2 system that a DNAse inhibitor is removed (202). The time required for the synthesis of the first new viral DNA has been determined as 6 to 7 min. with T2 where the incorporation of the unique viral constituent hydroxymethyl cytosine into DNA can serve as a marker (203). This result is in agreement with earlier results obtained by a number of other investigators using less sensitive procedures. The rearrangement of the host machinery can be considered largely completed by this time.

The increased enzymatic activities that occur within the first few minutes after T2 infection are shown in Figure 1. All the evidence presented by Flaks & Cohen (204) and Flaks *et al.* (205) on the appearance of deoxycytidine hydroxymethylase within 3 min. after infection is consistent with the conclusion that this enzymatic activity is caused by the formation of a new enzyme. This observation has been confirmed by Kornberg *et al.* (206), and Ebisuzaki, Figueiredo & Schlesinger (207) have observed similar reactions in

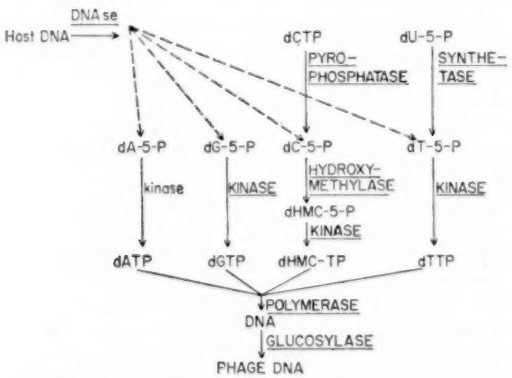

FIG. 1. Some of the increased enzymatic activities in *E. coli* after infection with bacteriophage T2. The kinase reactions probably occur in two steps and are catalyzed by two separate enzymes. The rate of conversion of deoxyadenosine-5′-phosphate to deoxyadenosine triphosphate does not increase after T2 infection.

The following abbreviations are used: dA-5-P for deoxyadenosine-5′-phosphate; dATP for deoxyadenosine triphosphate; dC-5-P for deoxycytidine-5′-phosphate; dCTP for deoxyribocytidine triphosphate; dG-5-P for deoxyguanosine-5′-phosphate; dGTP for deoxyguanosine triphosphate; dHMC-5-P for deoxyhydroxymethyl cytidine-5′-phosphate; dHMC-TP for deoxyhydroxymethyl cytidine triphosphate; dT-5-P for deoxythymidine-5′-phosphate; dTTP for thymidine triphosphate; dU-5-P for deoxyuridine-5′-phosphate.

protoplasts infected with urea-disrupted T2 phage. Cells infected with T2 (205) or T5 (206), which do not contain hydroxymethyl cytosine, do not produce this enzyme. Flaks & Cohen (208) and Barner & Cohen (209) have also reported that T2 and T5 infection causes the rapid appearance of an enzyme called thymine synthetase, which converts deoxyuridylic acid into thymidylic acid. The results of Amos & Magasanik (210) indicate that uridine is converted into thymidylic acid after T1 infection and support the conclusion that this is the main route of the thymidylic acid synthesis in phage infected cells.

Kornberg *et al.* (206) have found that the activity of the kinases (presumably two separate enzymatic reactions) that convert thymidylic to deoxyadenosine triphosphate (dATP) and deoxyguanosine-5-phosphate to deoxyguanosine diphosphate increases 20 to 45 times over that of the uninfected cell. The question whether these increased activities are caused by the increase in the amount of normal host enzymes or whether these are new enzymes has not been completely resolved. However, the report of Bessman & Van Bibber (211, 212) that deoxyguanylate kinase produced after infection has properties different from those normally found in the host suggests that in this case a new enzyme has been formed. The appearance of the kinase which converts hydroxymethyl deoxycytidine-5-phosphate to hydroxymethyl deoxycytidine triphosphate has been reported by Kornberg *et al.* (206) and Somerville & Greenberg (213). This activity is not found in uninfected cells or in T5-infected cells. The appearance after T2 infection of an active pyrophosphatase that cleaves deoxycytidine triphosphate has been reported, first by Kornberg *et al.* (206) and also by Koerner, Smith & Buchanan (214). Both groups have characterized this enzyme and suggest that it is newly synthesized after infection. The enzyme is highly specific and splits only deoxycytidine triphosphate. It can be suggested that the presence of this enzyme in T2-infected cells prevents synthesis of the normal cellular DNA and, by furnishing one of the intermediates for phage DNA, deoxycytidine-5-phosphate (see Figure 1), stimulates phage DNA synthesis (206, 214).

The level of deoxynucleotide polymerase activity also increases twelvefold after infection with T2 (206). It is noteworthy that this increase in activity can only be demonstrated when heated T2 DNA is used as a primer. No increase in activity was found when native calf thymus or T2 DNA were used. Kornberg and his co-workers (206) have also shown that the glucose found in T2 DNA is introduced after the DNA has been formed by a glucosylase using uridine diphosphate glucose. Loeb & Cohen (215) report that all the glucose of T2 phage is furnished by glucose of the medium. In view of the fact that the glucose contents of T2, T4, and T6 are genetically determined, one might expect that three different glucosylating enzymes would be formed after infection with these three bacteriophages.

The appearance of a number of enzymatic activities after infection with either T2 or T5 and probably other phages explains the effect of chloram-

phenicol reported in these and similar systems by Rosenbaum & Preston (216), Crawford (217), and Tomizawa (218). If chloramphenicol is added early enough it prevents DNA synthesis, but if it is added after these new enzymes appear the DNA is formed at the usual rate and is apparently normal.

An interesting effect of the antibiotic Mitomycin C, has been reported by Sekiguchi & Takagi (219) and Shiba *et al.* (220). In uninfected *E. coli* B, Mitomycin C stops DNA synthesis, but the synthesis of RNA and protein continues. Infection of bacteria by T2 in the presence of Mitomycin C relieves the blockage to DNA synthesis, and T2 is produced, but in reduced yield. These authors suggest that an alternate pathway for DNA synthesis is created by an infection which is no longer affected by Mitomycin C. In view of the new and increased enzymatic activities that are now known to appear upon infection, the relief of inhibition of DNA synthesis is not unexpected.

The mechanism by which phage infection initiates the production of new enzymatic activities, phage proteins, and phage DNA is not known, but there is some evidence that RNA is involved in some of these processes. The work of Volkin, Astrachan & Countryman (221), Astrachan & Volkin (222, 223) and Countryman & Volkin (224) has shown that infection with T2 or T4 causes an active metabolic turnover of a special small fraction of the host cell RNA without net synthesis. It is of interest that the RNA formed has base ratios similar to those found in the phage DNA. Chloramphenicol added at the time of infection inhibits the RNA breakdown in the T2-infected cells but not the synthesis of the new RNA. After DNA synthesis starts, addition of chloramphenicol does not prevent RNA breakdown. Watanabe, Kiho & Miura (225) have reported similar results in experiments with chloramphenicol. These experiments support the view that RNA serves as an intermediate between DNA and protein synthesis. Similar conclusions have been reached by Jeener (226, 227) and Jerne & Maaløe (228) in experiments on the effects of RNAse on phage-infected cells. The addition of RNAse inhibits phage protein synthesis in T4-infected cells and in induced lysogenic *Bacillus megatherium* cultures. Both groups conclude that a specific RNA is involved in phage protein synthesis.

Maturation of phage in infected cells.—The sectioning of infected cells has been the only direct and successful approach to the morphological problems involved in phage maturation. Kellenberger, Séchaud & Ryter (229) have shown that, 10 min. after infection with T2, the fibrillar DNA filling the cell begins to condense into dense head-shaped particles, which are some 20 per cent smaller than those of the finished virus. These workers suggest that a specific substance, "the condensation principle," is responsible for the condensation of the DNA. This compound may be the internal head protein or the polyamines. Once the condensation of the DNA has occurred, the appearance of the head membrane around the DNA can be observed. Some similar observations have been made by Mercer (230). The assembly of the tail

structure has not as yet been seen, but it is assumed that it is formed progressively on the immature phage.

Protein synthesis in disrupted host cells initiated by T2 DNA.—Brown & Brown (231) have reported that DNA isolated from T2 can cause the production of a phage tail protein in disrupted host cell protoplasts. If confirmed, these experiments would not only be the first report of the synthesis of a viral component but also of an effect of DNA in a broken cell system. The host cells were converted into protoplasts and osmotically shocked. These disrupted cell fragments could incorporate C^{14}-isoleucine into protein only if supplemented with an ATP-generating system, complete amino acid mixture, amino acid-activating enzymes from rat liver, polynucleotide phosphorylase, and all the riboside diphosphates. These requirements suggest that a net synthesis of protein is taking place and that a concurrent synthesis of RNA is required. When the T2 DNA that had been prepared by osmotic shock and column chromotography was added to the preparation, the synthesis of phage tail protein that was equivalent to 2×10^9 phage particles was detected by its reaction with antiserum to whole phage. DNAse treatment of the T2 DNA stopped the synthesis of this component. Although no detailed studies of the molecular weight of the active DNA have been carried out, it is noteworthy that other preparations of DNA that had been subjected to shearing forces (see above) during preparation were not active.

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THE BIOCHEMISTRY OF GENETIC FACTORS[1,2]

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The term genetic chemistry will be used in this chapter to refer to knowledge of the structure, function, replication, mode of expression, and modification of the genetic elements. The genetic elements are presently conceived to include double-stranded DNA as the genetic substance of the great majority of living organisms, single-stranded DNA as the genetic substance of certain bacterial viruses, and single-stranded RNA as the genetic substance of certain plant and animal viruses. Of necessity, discussion of the structure, replication, and expression (through the transfer of information) of these substances involves consideration of related ancillary structures, such as chromosomes and viral components. In addition to direct approaches, studies of the products of genetic action can be used as a basis for inference concerning the organization of the genetic substance, particularly when obtained under defined genetic circumstances.

Genetic Chemistry of DNA

Structural features.—The imposing body of evidence that the bulk of the DNA present in the great majority of living organisms has the two-stranded complementary helical structure first proposed by Watson & Crick (1) has been briefly reviewed (2).

The generality of this conclusion has been extended by x-ray diffraction studies (3), which demonstrate the gross structural identity of DNA from non-metabolizing forms (sperm, bacteriophage), from non-dividing cells (erythrocytes), from slowly dividing cells (liver, thymus), and from rapidly dividing cells (bacteria, leukemic leukocytes). Apparently the DNA molecules do not deviate from this form for any appreciable portion of a division cycle (unless an unstable altered state is postulated, which is transformed to the Watson-Crick structure during the process of extraction).

It is therefore of great interest that through physicochemical examination and chemical analysis the DNA in a small bacterial virus, ϕX 174, has been shown to consist of one strand of DNA (4, 5). This discovery demonstrates that one strand of DNA is adequate to carry hereditary information and raises important questions concerning the replication of genetic information (*vide infra*). The DNA content of this virus (1.7×10^6 in molecular weight units) is comparable to the RNA content of many plant and animal viruses,

[1] A survey of literature pertaining to this review was concluded on October 15, 1959.

[2] The following abbreviations are used: DNA for deoxyribonucleic acid; RNA for ribonucleic acid; TMV for tobacco mosaic virus.

and it may be that the mode of replication is more closely linked to that of such viruses than to that of conventional DNA.

The discovery of the single-stranded character of the DNA of ϕX 174 has clarified the unusual sensitivity of this virus to the decay of incorporated ^{32}P (6). The similar sensitivity to ^{32}P decay of the related bacteriophage S13 suggests that it also carries a single-stranded DNA. It is therefore of considerable significance that genetic recombination with strains of the S13 virus (7) can be demonstrated.

Attempts have been made to estimate the nucleic acid content of a genetic unit, or cistron (8), in bacteriophage and in DNA with transforming activity. An improved value (9) of Benzer's estimate of the fraction of the genetic map of T2 phage occupied by a particular cistron (r II B) is 0.75 per cent. If the DNA content of a T2 phage (200,000 nucleotide pairs) is equated to the genetic map, the r II B cistron will then contain approximately 1500 nucleotide pairs (molecular weight about one million).

More recently, attempts have been made to estimate the size of particular genetic determinants in pneumococcal DNA possessing transforming activity by measurement of the decline in activity accompanying molecular degradation by shear forces and by interpretation of this data in terms of a critical size of DNA necessary to contain the genetic information. Unfortunately, loss of transforming activity during degradation can be traced to a decline in absorption by the pneumococcal cells and possibly to a decline in the frequency of interaction with the host DNA, as well as to the postulated splitting of the essential genetic information, so that it is not possible to interpret the data in the way originally contemplated (10).

Lerman & Tolmach (11) have demonstrated that the decline of transforming activity brought about by deoxyribonuclease action follows "single-hit" kinetics, and that during the early stages of this process there is no decline in the absorption of such DNA by the bacteria. The "single-hit" kinetics imply that one break introduced by the enzyme within a critical mass of DNA can inactivate a particular factor. By an indirect calculation, based upon the rate of decline of viscosity of the DNA, these authors have evaluated the mean number of diester links broken per nucleotide, by deoxyribonuclease, at the time corresponding to one hit to the streptomycin resistance factor. They draw the conclusion that the scission of one diester bond within a DNA region of 500,000 M is sufficient to inactivate this factor.

Enzymes of DNA synthesis.—Following the discovery by Kornberg (12) of a DNA polymerase in bacterial cells, apparently similar enzymes have been described in extracts of rat liver cells (13, 14, 15, 16) and of ascites tumor cells (17). Further studies by the Kornberg group (18, 19) have shown that the DNA made *in vitro* by the action of this polymerase has a complementary (adenine = thymine; guanine = cytosine) composition and that the ratio [(adenine plus thymine)/(guanine plus cytosine)] of the product reflects that of the DNA employed as primer. In the absence of primer DNA,

the polymerase can synthesize a macromolecule composed exclusively of deoxyadenylic and thymidylic acid residues.

The polymerase will incorporate a variety of nucleotide analogues into DNA, always in accord with the complementary purine and pyrimidine pairing prescribed by the Watson-Crick structure. Thus, uracil, or 5-bromouracil, can substitute for thymine in the newly formed DNA, and only for thymine. Similarly, 5-methylcytosine or 5-bromocytosine will substitute for cytosine and hypoxanthine for guanine. The only apparent reason for the absence of uracil in DNA synthesized *in vivo* is the absence of a kinase to convert deoxyuridine-5′-monophosphate to the triphosphate.

The synthesis of DNA *in vivo* is obviously under strong control and closely correlated with the cycle of cell division. In this connection, it is of great interest that adult liver cells, while possessing significant amounts of DNA polymerase, deoxyadenylic acid kinase, deoxyguanylic acid kinase, and deoxycytidylic acid kinase are almost devoid of thymidylic acid kinase activity (13, 15, 16). During liver regeneration, the levels of thymidylic acid kinase activity and of DNA polymerase activity increase some tenfold during the period of rapidly increasing DNA synthesis 18 to 24 hr. after hepatectomy. It is of interest that a heat-stable, alcohol-soluble substance has been isolated from liver homogenate, which inhibits the uptake of ^{14}C-formate into DNA in bone marrow homogenates, even in the presence of deoxyuridine (20).

It has been known for some time that one of the more immediate effects of x-irradiation of cells is a pronounced decline in the rate of synthesis of DNA, and this observation has been confirmed by recent work (21, 22). Experiments by Billen (23), using a thymine-requiring strain of *Escherichia coli*, 15_{T-}, suggest that the biochemical mechanism for DNA synthesis may be a particularly sensitive target for x-irradiation. If *E. coli* cells of strain 15_{T-} are exposed to chloramphenicol (in the presence or absence of thymine) and then removed from chloramphenicol, DNA synthesis is blocked for a period of 30 min. It is suggested that the mechanism for DNA synthesis is either unstable or can be exhausted during this time. If, during the period in chloramphenicol, the cells are irradiated with x-rays (10,000r), DNA synthesis is never restored.

DNA turnover.—While there is general agreement concerning the absence of DNA turnover in rapidly growing cells (24, 25), Thomson *et al.* (26) and Price *et al.* (27) have shown a limited rate of turnover in slowly growing fibroblast cells or in resting bacteria. Halvorson (28) has provided similar evidence of nucleic acid turnover in resting yeast, by a process that is dependent upon the presence of an energy source.

Timing of DNA synthesis.—The failure to find any appreciable fraction of DNA molecules of intermediate density between "full-heavy" and "half-heavy" DNA in the ^{15}N-density-labelling experiment of Meselson & Stahl (29) indicated that the duration of synthesis of a particular molecule must

be brief compared to the bacterial generation time. While measurements of DNA synthesis in cultures synchronized by thermal shock or nutrient deficiency (30, 31) have indicated a brief duration for total cellular DNA synthesis, more recent experiments using cells synchronized by less perturbing means (32, 33) demonstrate that DNA synthesis is almost continuous throughout each cell generation. Radioautographic studies of the uptake of ^{3}H-thymidine (204) and the ingenious ^{32}P-decay experiments of McFall & Stent (34) lead to the same conclusion. It had been previously demonstrated that the death of bacteria by decay of incorporated ^{32}P is very largely the result of the decay of ^{32}P atoms incorporated into bacterial DNA. McFall & Stent demonstrated that exposure of an unsynchronized log phase bacterial culture to ^{32}P-containing medium for 5 min. (11 per cent of the generation time) resulted in sufficient accumulation of ^{32}P that 70 per cent of the cells died upon storage, while a 10 min. exposure brought about the death of over 95 per cent of the cells during ^{32}P decay. Thus, while the period of synthesis of each DNA molecule must be brief, the synthesis of the genetic complement of the cell must extend over nearly the entire division cycle. As a consequence, nearly all cells, though nominally haploid, must be partially diploid at any particular time.

DNA replication.—The density-labelling experiment of Meselson & Stahl (29) demonstrated that at each replication of DNA the elements of the DNA fraction examined, which were presumed to be double-stranded DNA molecules, split into two equal components, each of which was then paired with a newly formed structure to create elements of hybrid density. The half-size components were then conserved at each successive replication, and, indeed, the "heavy" and "light" components of the elements of hybrid density could be disassociated by heating. It was generally assumed that these persistent components corresponded to the single chains of the original double-stranded DNA molecule.

Cavalieri *et al.* (35) have now presented evidence that the DNA fraction examined by Meselson & Stahl is much more complex than a single two-stranded DNA molecule, and must be considered to be a chromosomal fragment or subunit. These authors confirm the report of Meselson & Stahl that the elements of this fraction can be split into two equal parts by heating. However, treatment of the elements of this DNA fraction with chymotrypsin or with deproteinizing agents resulted in a split of the original elements (molecular weight 11×10^{6}) into quarters (molecular weight 2.4×10^{6}). These quarter-sized pieces could still be split in two, to pieces of molecular weight 1.3×10^{6} by heating. Upon treatment of these smaller pieces with deoxyribonuclease, however, the kinetics of the decline of molecular weight suggested that these pieces were still two-stranded, while similar kinetic experiments suggested that the pieces of 2.4×10^{6} molecular weight were four-stranded or a pair of double helices.

If correctly interpreted, these experiments imply that DNA replication *in vivo* does not proceed by a separation of the two strands of the Watson-

Crick structure and by formation of new strands upon these by a complementary process, as originally suggested by Watson & Crick, but that, in some manner, old DNA double helices are wholly conserved, while new DNA double helices are completely new. Since this conclusion rests solely upon the kinetics of deoxyribonuclease digestion of components of 1.3×10^6 mol. wt. described above, some caution must be expressed. The interpretation of such experiments is difficult and it is not certain, for instance, that a single-stranded DNA structure could not form sufficient intramolecular hydrogen bonds to result in an apparent two-strand kinetics of degradation. In any event, it is clear that the DNA fraction examined by Meselson & Stahl is far more complex than was presumed, and the entire question of the mode of DNA replication must be re-examined.

It is not clear what relation the chromosomal fragments just described may bear to the DNA-protein isolated from *E. coli* by Zubay & Watson (36). The protein of this fraction (50 per cent by weight) is not of the histone class.

That other DNA preparations previously examined may have been more complex than has usually been assumed is indicated by the experiments of Hermans (37), which demonstrate that chymotrypsin treatment can reduce DNA preparations of varying molecular weight from various mammalian sources to a common size of about 6×10^6 mol. wt., and by the experiments of Shumaker (38), which indicate the existence of fractions with distinct and sharply defined sedimentation rates.

DNA and the bacteriophage chromosome.—While the above experiments suggest that the chromosome of bacteria is a complex nucleoprotein, more evidence has accumulated that the chromosome of bacteriophage appears to be exclusively nucleic acid. A small amount of a basic protein (39) accompanies the DNA into the bacterium, but inhibition of its synthesis (and of all other protein) by chloramphenicol after 10 min. of infection has no effect upon the rate of continuing phage-DNA synthesis (40). That such DNA, made in the absence of concomitant protein synthesis, is the phage chromosome is indicated by three lines of evidence: Hershey *et al.* (41) demonstrated a stimulation to genetic recombination by ultraviolet irradiation of the DNA during a period of chloramphenicol inhibition; secondly, Tomizawa (42) showed that lesions introduced by ultraviolet irradiation into DNA synthesized during chloramphenicol inhibition appear undispersed, as chromosomal lesions, in progeny phage formed after the release of inhibition; and thirdly, Brenner & Smith (43) and Litman & Pardee (44), reported that mutations can be induced in phage progeny by exposure of infected cells to 5-bromouracil (*vide infra*) while DNA synthesis is proceeding but protein synthesis is blocked by chloramphenicol.

This apparent difference in the chromosomal nature of bacteria and bacteriophage may be the basis for the interesting reports that the antibiotic mitomycin, which specifically blocks DNA synthesis (while permitting RNA and protein synthesis) in bacteria (45), does not block DNA synthesis (at comparable levels of antibiotic) in phage-infected cells (46).

Although chloramphenicol does not inhibit DNA synthesis once initiated in cells infected with T-even phages, this antibiotic does inhibit DNA synthesis at any stage of infection with the phage T5 (47, 48).

DNA and lysogeny.—It has been apparent for some time that in lysogenic cells the replication of the carried (or of superinfecting) phage (prophage) is repressed by some physiological mechanism. Jacob & Wollman (49) demonstrated that if, during a bacterial cross, a carried phage was introduced into a prophage-free cell, this inhibition was almost invariably released ("zygotic induction"). Borek & Ryan (50) have recently demonstrated the converse: if ultraviolet-irradiated, F^+, prophage-free cells are mated to prophage-carrying, F^- cells during the cross a factor is transferred from the F^+ to the F^- cells which releases this inhibition and provokes induction.

Chromosomal replication.—The elegant autoradiographic studies of the chromosomal distribution of ^{3}H-thymidine-labeled DNA after successive cell divisions, initiated by Taylor & Woods (51), Taylor (52), and Woods & Schairer (53), indicated that the DNA synthesized during one interphase was equally distributed among the daughter chromosomes after the following metaphase and was, in the absence of sister chromatid exchange, restricted to two of the four daughter chromosomes present after a second metaphase. This suggested that the original chromosome contained two equal DNA-containing subunits, which separated at each replication. These subunits could be the sister chains of the Watson-Crick double helices, organized in some manner along the entire length of the chromosome. Indeed, an analysis of the number of sister chromatid exchanges, with reference to the relative proportions of twin and single exchanges, led to the conclusion by Taylor (52) that the two DNA subunits of the chromosome are structurally different in some way that prevents direct reunion between them. Such a difference could be explained by the opposite polarity of the chains of the Watson-Crick structure. Chromosomal structures based upon these ideas have been proposed by Taylor (54), Schwartz (55), and Freese (56).

LaCour & Pelc (57, 58) have claimed that the results just described are in part an artifact resulting from the use of colchicine and that, in the absence of colchicine, distributions of radioactivity other than those described by Taylor—such as the complete lack of label in one chromosome after one mitosis, or general labelling of all four chromosomes after the second metaphase—can be found. They reject the suggestion of Woods & Schairer (53) that their results are a consequence of the failure to use only chromosomes sufficiently close to the photographic emulsion to permit penetration of the weak tritium β-rays.

Studies of the timing of DNA synthesis have revealed that in some plant species (*Crepis*) a wave of DNA synthesis begins at both ends of each chromosome and proceeds toward the centromere (59). However, this does not appear to be true of other species (*Bellevalia*), in which the chromosomal sites of initiation of DNA synthesis are more random (51).

In the macronucleus of certain protozoa, a wave of DNA synthesis ap-

pears to begin at the two ends and move toward the center (60). In explants of rat heart cells in tissue culture, autoradiographic studies indicate that DNA synthesis begins with the DNA adjacent to the nucleolus and spreads from there (61). Lima-de-Faria (62) has shown that during the meiotic division of grasshopper spermatocytes, DNA synthesis in the sex chromosomes begins and ends distinctly later than DNA synthesis in the autosomes. The mechanisms for the control of these processes are quite unknown.

The expression of the DNA information: Coding.—It is generally thought that the genetic information contained in DNA is in some manner coded into the nucleotide sequence. This information must ultimately be employed to direct the construction of the polypeptide chains of protein molecules. It is usually proposed that RNA molecules serve as intermediate templates between the information contained in DNA and the peptide chains constructed according to this information. However, Pardee (63) has recently suggested, from a study of the kinetics of the formation of β-galactosidase upon introduction of the appropriate gene into an appropriate bacterium during a bacterial cross, that the DNA may participate directly in protein synthesis.

The discovery of a single-stranded DNA in certain bacterial viruses (5) and *in vitro* production of bacteriophage heterozygotes by chemical mutagens (*vide infra*) demonstrates that one strand of DNA is sufficient to convey genetic information. It is not known whether two-stranded DNA is "read" as a double helix or whether one or both chains are "read" individually. If the former hypothesis is correct, the problem arises as to the direction in which the double helix is to be "read." Golomb *et al.* (64) have considered the construction of codes that contain, intrinsically, a sense of one direction.

The crux of the coding problem is the manner of translation of a series of nucleotides into a sequence of amino acids. For simplicity it is usually assumed that there is, corresponding to each amino acid, a linear sequence of nucleotides in DNA and in the RNA derived therefrom.

Recent studies on the heterogeneity of DNA molecules within and among various bacterial species have cast doubt upon this coding concept. It is known that the over-all nucleotide composition of the DNA of various bacterial species varies widely (65, 66), while the over-all nucleotide composition of the RNA of these strains is much more uniform (66). Recently, work in two laboratories has demonstrated that the nucleotide composition within individual DNA molecules determines the denaturation temperature and the density of these molecules (67, 68, 69, 70). Thus, measurement of the spread of denaturation temperatures or of the spread of the densities (in an equilibrium density gradient) of the DNA molecules of a particular preparation enables the establishment of upper limits for the spread of nucleotide composition among the molecules of the preparation. In this way it has been shown that the spread of composition of the DNA molecules within various bacterial species is very slight, whereas the mean nucleotide composition of the various DNA molecules of different species varies so widely that there are among the set of DNA molecules of certain species no molecules with the same composi-

tion as those of the set found in other species (no overlap of composition). Despite these observations, the RNA molecules of such species do not differ widely in composition, and the various species display very nearly the same complement of enzyme activities. The RNA composition also remains constant through the various phases of a bacterial culture (71).

No widely accepted explanation has been advanced for these observations, although various possibilities, such as the presence of appreciable redundancy in the DNA code (e.g., the code may be using only two symbols instead of four as has been assumed) or the non-generality of any particular code, have been suggested.

In contrast to these conclusions are observations on RNA synthesis during bacteriophage infection; the composition of the RNA produced changes abruptly and closely parallels the composition of the DNA of the infecting particle (72, 73). This shift in pattern of RNA synthesis upon infection can be prevented by the addition of chloramphenicol (74).

The expression of the DNA information: RNA synthesis in cells.—The manner of synthesis of RNA in cells continues to be a major enigma. Zubay (75) has speculated as to the way in which a two-stranded DNA might direct the synthesis of RNA. The experiments of Mandel *et al.* (76) and Hurwitz *et al.* (77) suggest that the precursors of RNA may be the ribonucleotide triphosphates. Allfrey & Mirsky (78) have demonstrated that removal of DNA from isolated thymus cell nuclei destroys the capacity of such nuclei to incorporate adenylic acid into RNA. But they have also shown that this capacity can be very effectively restored by the addition of non-specific polyanions such as polyethylenesulfonate.

The discovery of a wide variety of minor constituents in various RNA fractions has also complicated consideration of the transfer of information from DNA through RNA to protein. These components include 5-ribosyluricil (79, 80, 81, 82, 83, 86), 5-methylcytosine (83, 84), thymine (85, 86), 2-methyladenine, 6-methylaminopurine, 6-dimethylaminopurine, 1-methylguanine, 2-methylamino-6-hydroxypurine, 2-dimethylamino-6-hydroxypurine (83, 85, 86, 87), and a set of nucleotides with an unusual sugar, 2′-O-methyl ribose (88). These unusual nucleotides appear to be concentrated in the "soluble RNA" fraction which contains the RNA active in amino acid transfer. Smaller amounts of these nucleotides are found in microsomal RNA, while the RNA of two plant viruses appears, interestingly, to lack them entirely.

A variety of attempts has been made by use of nucleate and enucleate cells or autoradiographic means to localize the site of RNA synthesis and to study the pattern of flow of RNA within cells. The results seem to indicate that RNA synthesis is largely a nucleolar function and that RNA flows from the nucleolus to the cytoplasm.

Careful experiments using isotope dilution analysis appear to have established that there is no net synthesis of RNA in enucleate *Acetabularia* (89). Plaut & Rustad (90) have reported incorporation of labelled uracil and orotic

acid into the RNA of the enucleate amoeba; however, Prescott (91) has reported that such incorporation does not occur if the amoeba are well starved before enucleation and that this is presumably an incorporation by viable microorganisms within food vacuoles.

Several autoradiographic experiments (51, 92, 93, 94), employing tritiated thymidine or uridine, have shown that a strong accumulation of radioactivity is first observed in the cellular nucleolus, whereas the appearance of radioactivity in the cytoplasm is considerably delayed. Pulse experiments indicate that the radioactivity in the nucleolus is gradually transferred entirely to the cytoplasm. This transfer cannot be diluted by addition of nonradioactive nucleosides and so presumably consists of polynucleotide materials.

In an elegant experiment that employed autoradiography of centrifuged *Neurospora hyphae* which had been fed tritiated uridine (92), the distribution of radioactivity was followed among the stratified cellular constituents (cytoplasm, nucleus, mitochondria, microsomes, glycogen granules). In pulse experiments, activity first appeared in the nuclear layer and then, at the expense of the nuclear label, in the microsomal layer (and to a lesser extent in the mitochondrial layer).

The relationship of RNA synthesis to the presumed DNA template is unclear. In some organisms (*Tradescantia*) there is very little RNA synthesis during DNA replication, but in others (*Vicia faba*, *Drosophila* salivary gland) DNA and RNA synthesis can proceed simultaneously (51). Goldstein & Micou (95), using human amnion cells in tissue culture, have recently demonstrated, with a very brief pulse of tritiated thymidine, that an RNA component is rapidly synthesized in the chromatin and then quickly transferred to the nucleolus and may in this case be the origin of much of the nucleolar RNA. Such a component may have been missed in other radioautographic experiments, but this result seems to be in direct contradiction with that of Woods & Taylor (51) with *Drosophila* larvae.

An interesting aspect of the recent radioautographic experiments has been the discovery of thymidine-containing bodies in the cytoplasm. The parabasal body at the base of trypanosome flagella incorporates tritiated thymidine (96). An appreciable uptake of thymidine into the chloroplasts of *Spirogyra* was observed (97). This result is of interest in connection with recent experiments that indicate the genetic continuity of chloroplasts in *Euglena* (98). The significance of the widespread thymidine incorporation into the cytoplasm of amoeba (99) is unclear in view of the possible presence of viable organisms in food vacuoles (91).

The expression of the DNA information: the problem of differentiation.— The invariance of the amount of DNA in the diploid nucleus of various types of differentiated cells under normal conditions has been well established. Recent chromatographic investigations (100, 101) have demonstrated that the elution patterns of DNA prepared from various differentiated cells are identical; this contradicts an earlier report (102).

Some suggestions of an altered DNA metabolism accompanying differentiation have been derived from autoradiographic experiments. Pelc & LaCour (103) have observed a marked uptake of thymidine into cells of *Vicia faba* roots at a stage when they are known to have finished cell division and are beginning to elongate. Previous studies have demonstrated that the DNA content of cells at this stage is not increasing, and radioautographic studies with ^{32}P or ^{14}C-adenine do not reveal any marked uptake into these cells.

McMaster-Kaye & Taylor (104) have shown that, at an early stage of differentiation of the *Drosophila* salivary gland, the amount of RNA in the nucleus rapidly drops by two-thirds, while the amount of DNA increases to just the same extent. Autoradiographic and spectrophotometric studies of the chromosomal puffs (105, 106) of *Drosophila* and *Rhynschosciara* indicate a considerable variability, in that some puffs actively synthesize DNA, while others appear to be synthesizing and turning over RNA.

The function of the nuclear histones remains obscure, but as evidence of the complexity of these substances is accumulated, the possibility that they may play a role in differentiation is enhanced. Improved methods of fractionation of histones have been developed (107, 108, 109), and Neelin & Connell (107) have demonstrated by electrophoresis on starch gel the presence of 16 distinct components in chicken erythrocyte histone.

Mauritzen & Stedman (110) have reported on an unusual histone of high arginine content, which is present in the nuclei of fowl erythrocytes but not in nuclei of other types of fowl cells. They also found small but significant differences in the amino acid compositions of the β-histone fractions from fowl erythrocyte nuclei, fowl spleen nuclei, and fowl liver nuclei.

Woernley *et al.* (111) have demonstrated an apparent difference in the binding to protein of a fraction of the DNA of ascites tumor nuclei as compared to similarly prepared thymus nuclei. Goldstein (112) has made the very interesting observation that, if amoeba nuclei, labelled by growth of the amoeba in ^{35}S-methionine, are transferred to unlabelled amoeba, the label in large part leaves the transferred nucleus to enter the cytoplasm and is then reconcentrated into both the transferred and the host nuclei. Goldstein believes that this label may represent a protein component which accompanies the RNA into the cytoplasm and is then recycled.

Spectrophotometric evidence has accumulated for apparent deviations from the diploid content of the DNA in the cells of certain organs under special conditions. Lowe *et al.* (113) have demonstrated a drop of 20 per cent in the DNA content of diploid, tetraploid, and octaploid cells of rat liver upon treatment with cortisone. Leeman (114) has reported an increase of 11 per cent in the DNA content of nuclei of the adrenal medulla in rats kept at 4°C. for two weeks as compared with controls. It is not clear whether these effects represent an actual change in the DNA content of these nuclei or possibly changes in the solubility of the DNA during fixation procedures or in the reactivity of the DNA to the Feulgen procedure.

Stich & Emson (115) have reported aneuploid amounts of DNA in the cells of a considerable number of human carcinomas examined at metaphase or early anaphase to minimize the possibility of error attributable to DNA synthesis.

Koenig (116, 117) has demonstrated by autoradiography an uptake of thymidine into the nuclei of non-dividing oligodendrocytes of the central nervous system of adult cats, but the significance of this is uncertain until information is available concerning the possibility of polyploidy in such cells.

Changes in the over-all nucleotide composition of the RNA of *Chlorella* cells have been reported, during starvation, by Newmark & Fujimoto (118), and in etiolated cells accompanying chloroplast formation by Brawerman & Chargaff (119). Levenbook *et al.* (120) have demonstrated an interesting increase (from 25.2 to 29.8 mol per cent) in the molar proportion of adenine in the RNA of XXY eggs of *Drosophila* as compared with the composition of the RNA of XX eggs.

A multiplicity of electrophoretically different forms of the crystalline enzyme lactic dehydrogenase has been reported from various tissues of the pig and other mammals by Markert & Møller (121). One tissue may contain several forms, but the relative proportions of different forms vary in different tissues. Embryonic tissue may often contain a different pattern, both quantitatively and qualitatively, from that of the corresponding adult tissue. The origin and genetic basis of these various forms of the enzyme (similar results are reported for isocitric dehydrogenase and malate dehydrogenase) are not known.

The expression of the DNA information: bacteriophage infection.—Upon infection of a bacterium by a T2 phage a cluster of new enzymes is produced. These include a deoxycytidylate hydroxymethylase, which converts deoxycytidylic acid to 5-hydroxymethyldeoxycytidylic acid (122, 123), a hydroxymethyldeoxycytidylate kinase, which converts 5-hydroxymethyldeoxycytidylic acid to the triphosphate (124, 125), and a deoxycytidylic triphosphatase (124, 126), which converts deoxycytidine triphosphate to the monophosphate by hydrolysis of the pyrosphosphate link. The presence of these enzymes prevents the accumulation of any deoxycytidine triphosphate, while providing 5-hydroxymethyldeoxycytidine triphosphate and thereby explains the complete substitution of cytosine by 5-hydroxymethylcytosine in the DNA of the T2 phage.

Another interesting enzyme which appears in T2 infection glucosylates a fraction of the hydroxymethyl groups of 5-hydroxymethylcytosine, after incorporation of the nucleotide into macromolecular DNA (124). The glucose is transferred from uridine diphosphate glucose.

There are also considerable increases in DNA-polymerase activity and in the levels of deoxyguanylic acid kinase and thymidylic acid kinase activity in both T2 and T5 bacteriophage infection (124). Evidence indicating that the deoxyguanylic acid kinase activity in T2 infection (127) and the thymi-

dylic activity in T5 infection (128) have different properties with respect to ion activation and temperature sensitivity, respectively, than those of the similar enzymes of the uninfected cells suggests that these activities represent the synthesis of new enzymes derived from information provided by the infecting DNA rather than augmentation of the levels of bacterial enzyme activity.

Mutation.—Changes in the DNA of organisms or viruses can result in the production of inheritable alterations of genotypic and phenotypic characters, or mutations. Such changes can be brought about by treatment with ultraviolet radiation, by the incorporation of nucleotide analogues, by the action of certain dyes such as proflavin, by interference with DNA synthesis as in thymine starvation, by the action of heat, and by the direct chemical effect of deaminating or alkylating agents.

The mode of production of mutations by ultraviolet irradiation is still obscure. Earlier work had suggested a direct effect of photons absorbed by the nucleic acid. Doudney & Haas (129, 130) suggest that mutation is a consequence of the incorporation of radiation-altered nucleotide precursors into nucleic acid (specifically RNA) following the irradiation. They propose that these altered precursors must first be "stabilized" after irradiation by a reaction which requires the presence of amino acids and the possibility of protein synthesis. If neither amino acids nor a nitrogen source are present, or if chloramphenicol is added, these precursors are removed by some energy-dependent process, which can in turn be blocked by the addition of dinitrophenol. "Stabilization" of the precursors, however, does not immediately result in "fixation" of the mutation, as the addition of chloramphenicol at any time up to 30 min. after irradiation can markedly depress the final number of mutants produced. Beginning at 30 min. and coincident with the post-irradiation development of RNA synthesis, the ultimate level of mutants becomes resistant to the "chloramphenicol challenge" and is totally resistant after 75 min. The postulated modified RNA is presumed to result somehow in the production, ultimately, of altered DNA.

The evidence in favor of this hypothesis consists of the effects of various pre- and post-irradiation treatments (addition of nucleotide precursors at various times, the influence of post-irradiation nitrogen or phosphorus starvation, exposure to chloramphenicol, 5-hydroxyuridine, dinitrophenol, etc.) upon the final level of auxotrophic mutants produced by a given irradiation, combined with measurements of DNA, RNA, and protein synthesis after irradiation.

The argument is self-consistent, but does not take into consideration the possibility of direct repair of ultraviolet damages as an alternative explanation (*vide infra*). A critical experiment is needed to distinguish between the alternative possibilities that the primary injury is to the DNA or to the cytoplasmic components.

Wyss & Ray (131), using synchronous cultures of *Escherichia coli*, have

demonstrated two peaks of sensitivity to mutation production by ultraviolet irradiation during each division cycle.

Litman & Pardee (133) have shown with 5-bromouracil in bacteriophage that the incorporation of base analogues into DNA can induce mutations [for a review of analogue incorporation see Matthews (132)]. Similar results have been obtained with 2-aminopurine (134). By means of the detailed genetic mapping possible in the r II cistron of T4 bacteriophage, Freese (134) and Benzer & Freese (135) have shown that the maps of mutant sites produced by these two agents are different and that each differs from the map of spontaneous mutants or the map of proflavin-induced mutants (136). There appears to be a limited number of distinct sites that are particularly sensitive to the action of each mutagen ("hot spots") as well as a larger number of sites of lower sensitivity to each mutagen. No coincidences were observed between the map sites of 55-proflavin-induced mutants and 67 5-bromouracil-induced mutants.

5-Bromouracil and 2-aminopurine are believed to act as tautomeric mutagens (137) and to bring about the replacement of adenine-thymine nucleotide pairs by guanine-cytosine pairs, or vice versa. As such, one would expect these agents to be highly effective in producing reversions of the mutations which they had produced, and Freese (134) has demonstrated this to be the case. Curiously, 2-aminopurine is particularly effective in producing reversions of 5-bromouracil-induced mutations and vice versa.

The nature of the mutagenic action of proflavin is unknown, as is the manner of origin of the spontaneous mutants. It is of great interest, however, that spontaneous and proflavin-induced mutants cannot be reverted by the action of 2-aminopurine or 5-bromouracil, although such mutants do revert spontaneously, at a low rate. Freese (134) has suggested that such mutations may represent replacement of a purine-pyrimidine nucleotide pair by a pyrimidine-purine nucleotide pair.

The enhancement of mutation by thymine starvation of thymine-requiring bacteria was first reported by Coughlin & Adelberg (138) and has been confirmed by Kanazir (139). A fourfold increase in the number of mutants was observed during the 30 min. period of starvation prior to the onset of "thymine-less death." Appropriate reconstruction experiments showed that this did not simply represent a selection of pre-existing mutants that were particularly resistant to thymine starvation. Greer & Zamenhof (140) have demonstrated that exposure of *E. coli* cells to 60° for 4 hr. yields a variety of mutants, which they attribute to a loss of purines from DNA.

The mutagenic action of nitrous acid was first demonstrated with TMV RNA by Mundry & Gierer (141) (*vide infra*), but has since been extended to bacteriophage (142), to bacteria (143, 144), and to transforming factor (145). Vielmetter & Wieder (142) have demonstrated that both the lethal and mutagenic action of nitrous acid on T2 bacteriophage follow "one-hit" kinetics. A variety of mutant types was observed. Many of the mutants are appar-

ently heterozygous, as in single infection they give rise to an equal number of wild type and mutant progeny. Such mutants have presumably been altered in only one DNA chain. Other mutants, although arising by a one-hit process, are apparently homozygous. To explain these results, the authors postulate three types of event: (*a*) a one-hit event that totally inactivates by preventing separation of the DNA chains; (*b*) a one-hit event that mutates one chain and results in a heterozygote; and (*c*) a one-hit event that is lethal to one chain and results in either wild type or mutant, depending upon the status of the other chain.

In contrast, both inactivation and mutation production in bacteria (presumably mono-nucleate) are "two-hit" events (143, 144). Despite this conclusion, no heterozygotes were observed, and Kaudewitz concludes that to obtain a detectable mutation in bacteria, both DNA strands must be altered. It is not obvious how this result is to be reconciled with the bacteriophage data.

Nitrous acid is a potent mutagen of transforming factor (145), and *in vitro* mutations that confer upon the DNA the ability to transform to optochin, streptomycin, or aminopterin resistance are readily obtained in large numbers. There is a simultaneous process of inactivation, and the quantitative aspects of this system can only be evaluated after more is known of the effects of this treatment upon absorption of the DNA by the bacteria.

Loveless (146) has demonstrated the production of bacteriophage mutants by treatment *in vitro* with alkylating agents which act to ethylate. Very curiously, while able to inactivate phage, methylating agents were totally inactive as mutagens.

Mention should be made of the extraordinary observations by Brink (147, 206) and Coe (207) concerning the hereditary changes in certain genes by passage through a generation in which they are accompanied by a particular allelic gene on the homologous chromosome.

Suppressor mutations are genetic alterations which act partially or wholly to undo the phenotypic effect of a previous mutation known to be in a different region of the genetic map. It has usually been considered that such mutations act indirectly (148), e.g., the original mutation may produce a modified enzyme, which is inactivated at normal cellular levels of some ion, while the suppressor acts to restore activity by lowering the cellular concentration of the ion concerned. However, Crawford and Yanofsky (149, 150) have demonstrated by chromatographic means that a particular suppressor of a tryptophan-requiring mutant acts by a direct conversion of the altered protein component made by the mutant, of the enzyme tryptophan-synthetase, into a functional and apparently normal protein. The suppressor is not a new gene for the synthesis of the normal protein, as its action is specific for this particular mutation, and it is not effective with other mutations known to affect the same protein. The concept that some genes can act so as to modify the structure of a protein determined by a different genetic locus is of considerable importance.

Repair of genetic lesions.—Increasing evidence is available that cells possess a mechanism for the repair of various types of lesions to DNA, particularly those inflicted by exposure to ultraviolet radiation. The most direct experiments are those of Stuy (151). Ultraviolet irradiation of a streptomycin resistant strain of *Hemophilus influenzae* to about 1 per cent survival was found to reduce the transforming ability (for streptomycin resistance) of DNA extracted from the irradiated cells by 40 per cent. The rate of DNA synthesis in such cells was reduced about 50 per cent for 40 min. after irradiation, after which it resumed a normal level. During this 40 min. period, however, the ultraviolet damage done to the DNA was repaired and the transforming activity per unit of DNA was restored to normal. (Sixty minutes after irradiation, the transforming activity of the extracted DNA again declined, followed by a decline in DNA content of the cells and in turn by cell lysis.)

Harm (152) has demonstrated that T4 phage carries a gene which, if uninjured, during infection can bring about the repair of a major fraction of ultraviolet lesions induced in T4 by ultraviolet irradiation or the repair of ultraviolet lesions induced in irradiated related phage if they are employed in a mixed infection with T4 phage. The class of lesions subject to repair by this mechanism appears to be the same class that is susceptible to photoreactivation.

Gillies & Alper (153) have shown that, if a culture of *E. coli* is given an ultraviolet dose of such a strength that less than 1 per cent would survive if plated directly on nutrient agar, the survival can be raised to over 90 per cent if the cells are incubated for an hour on nutrient agar and then placed on agar containing chloramphenicol for 2 hr., before plating. Radiation doses of this magnitude generally introduce a block of unknown nature to DNA synthesis in bacteria (154). This block can be repaired, but protein and RNA synthesis must be permitted in the period immediately following irradiation. Treatment at this first stage with chloramphenicol or 5-hydroxyuridine or deprivation of amino acid- or pyrimidine-requiring auxotrophs of their required nutrients will prevent resumption of DNA synthesis (155, 156, 157). It is not known whether this repair process involves direct repair of lesions in DNA [a similar requirement has been found for the resumption of DNA synthesis after treatment of bacteria with nitrogen mustards (158)]. Treatment with chloramphenicol for a period after the resumption of DNA synthesis is believed to allow restoration of a proper ratio of DNA to other components of the cell and thus to prevent death by "unbalanced growth" (157).

It seems conceivable that the failure of methylating agents to induce mutations in bacteriophage (146) may be a consequence of the presence within cells of a mechanism that demethylates such modified DNA.

Genetic Chemistry of RNA

Infectious RNA.—Since the discovery of the infectivity of the free RNA of TMV by Gierer & Schramm (159) and Fraenkel-Conrat (160), the RNA

preparations of three other plant viruses—tobacco ring spot (161), tomato bushy stunt (162), and turnip yellow mosaic (163)—have been shown to be infective at about the same relative level of infectivity. (RNA is about 0.1 to 1 per cent as infective per mole when free as when it is used in the intact virus.) Infective RNA has also been obtained from leaves infected with necrotic ring spot virus (164).

Infective RNA preparations have been made from either purified preparations of, or tissues infected with, a wide variety of animal viruses, including several encephalitis viruses (165, 166, 167, 170, 171, 172): Semliki Forest virus (175), poliomyelitis virus (168), influenza virus (169), and the virus of foot and mouth disease (173, 174, 205). In several instances the activity of the final phenol-treated extract was not affected by treatment of the tissue homogenate with ribonuclease, and, in these cases, the RNA is presumably derived from intact virus particles. In other cases, treatment of the virus with cold phenol does not release RNA, so the observed activity in phenol-treated extracts of tissue homogenate must have been present as free RNA in the homogenate, e.g., treatment with phenol at 50°C. is necessary to release RNA from western equine encephalitis virus (172).

Physicochemical studies of the infective RNA from TMV (176, 177, 178, 179) indicate the presence of a major macromolecular component of S_{20} about 30 and molecular weight 1.9 to 2.0×10^6, which is apparently the infective agent. RNA of similar sedimentation properties has been obtained from eastern equine encephalitis virus (170) and from polio virus (165).

Fraenkel-Conrat & Singer (180) have recovered 30 to 80 per cent of the initial infectivity of TMV preparations, dissociated to RNA and protein, by improved reconstitution procedures. Attempts to produce virus particles containing more than one type of RNA by reconstitution in the presence of RNA preparations from two different TMV strains were unsuccessful.

Reddi (181) has demonstrated small but significant differences in the proportions of various di- and trinucleotides produced from the RNA preparations of different TMV strains by the action of pancreatic ribonuclease.

Mutation of RNA.—Mundry & Gierer (141) have reported the production of mutants of TMV, recognized by their ability to produce necrotic lesions on Java-type tobacco plants, by treatment of either whole virus or free RNA with the deaminating agent, nitrous acid. The number of mutants produced at first increases linearly with time of exposure, indicating a "one-hit" process. The absolute number of mutants rises to a maximum of over 20 times the background and then declines because of accompanying processes of inactivation. At 1/e survival, 6 per cent of the survivors are mutant, so the probability for production of the mutant is estimated to be 6 per cent of that for inactivation. Estimates from measurements of the numbers of deaminated nucleotides, obtained by degradation of the RNA after prolonged deamination, suggest that the early rate of deamination is such that deamination of any one of about 3000 of the 6000 nucleotides must be lethal, while deamination of any of another set of about 180 nucleotides (6 per cent of 3000) must

give rise to this particular group of mutations. Many other survivors show other aberrant symptoms.

Bawden (182) has questioned this work by demonstrating that the number—and the appearance—of necrotic lesions caused by a given inoculum of a virus is strongly influenced in a non-linear manner by the presence in the inoculum of added amounts of a virus that does not produce necrotic lesions. Thus, if the original virus preparation of Mundry & Gierer contained a small fraction of necrotic lesion-producing mutants, the increase in the apparent number of mutants obtained after treatment with nitrous acid could simply represent an increased opportunity for the expression of such mutants consequent to the inactivation of large numbers of the wild-type virus. (It is also possible that the necrotic variants are more resistant to the action of nitrous acid than the wild-type virus, although this explanation need not be invoked.) The first-order kinetics could conceivably be a consequence of first-order inactivation.

While Bawden's criticism casts considerable doubt on the quantitative aspects of the Mundry & Gierer experiment, it seems unlikely that the entire apparent development of mutants, in large numbers at low level of inactivation, is merely a consequence of such viral interactions. Nitrous acid has clearly produced mutants in bacteriophage (142), studied in single infection, where such interactions do not exist, and there seems to be no reason why it could not similarly produce mutants in infective RNA.

Francki & Matthews (183) demonstrated that the incorporation of thiouracil into TMV-RNA reduced the specific activity of the virus by 55 to 80 per cent, but they did not inquire into the possible induction of mutations.

Intracellular events in RNA virus synthesis.—Siegel *et al.* (184) proposed as an inference from studies of the changes of the ultraviolet sensitivity of TMV infected leaves with time after infection, that one of the first steps of infection is the separation of the virus RNA from its protein coat. In this case, the appearance of intact virus would be expected sooner following infection with free RNA, than following infection with intact virus, and this has been observed (185, 186).

Engler & Schramm (187) have assayed, at various times after infection, the total amount of infective RNA obtainable from the leaves and the amount obtainable after incubation of the homogenate at 37° before phenol extraction. The difference is the amount of free RNA which is destroyed by enzymes during incubation, while the assay after incubation indicates the amount of RNA enclosed within intact virus. Thus, the appearance of free RNA precedes the appearance of mature virus by some 10 hr. (at 23°C.), but, once initiated, virus assembly soon overtakes RNA production and eliminates the pool of free RNA.

It is possible by illumination of the infected leaves to obtain a reactivation of TMV-RNA after ultraviolet irradiation of free RNA, but not of irradiated intact TMV or of TMV-RNA that has been extracted from the virus after irradiation (188).

Attempts to obtain mixed infection by strains of plant viruses continue to be fruitless, and it has been suggested by Siegel (189) that an exclusion mechanism is operative. This does not appear to be the case for RNA-containing animal viruses since for recombination has been observed with influenza virus (190, 191, 192) and multiplicity reactivation has been observed with ultraviolet-irradiated polio virus (193).

Conflicting reports have appeared concerning RNA, protein, and DNA metabolism in polio-infected HeLa cells (194, 195). Mueller *et al.* (196) have made the important discovery that homogenates of chick embryos infected with fowl plague virus can incorporate labelled amino acids into the S antigen of the virus (196). Brown & Stewart (197) have reported that treatment of foot and mouth disease-infected pig kidney cells with proflavin has little effect on the synthesis of the viral RNA but strongly inhibits synthesis of the intact virus. Similar results have been previously obtained with bacteriophage.

Cytological studies of sections of TMV-infected plant cells, using a fluorescent derivative of TMV antibody, have indicated that the first appearance of TMV antigen is in the cytoplasm near to the nuclear membrane (198). The antigen then spread throughout the cytoplasm but never appeared in the nucleus or chloroplasts. Similar antigenic studies of cell fractions obtained by homogenization indicated a high concentration of TMV antigen in the microsomes.

Using a similar fluorescent antibody technique on embryos infected with polyoma virus, Henle *et al.* (199) obtained a distinctly different result, in that the virus antigen first appeared as granules in the nucleus and later appeared in the cytoplasm.

RNA virus structure and synthesis.—By careful attention to the ionic conditions of sedimentation and diffusion, two groups (200, 201) have been able to obtain physicochemical evidence for the long postulated subunit of TMV protein of 17,800 mol. wt. Finch & Klug (202) have carried out an elegant x-ray diffraction analysis of polio virus and conclude that the protein shell is composed of 60 identical subunits of molecular weight about 80,000, arranged in icosahedral symmetry. All three small viruses so far examined (polio virus, tomato bushy stunt virus, and turnip yellow mosaic virus) possess this icosahedral symmetry, and Finch & Klug suggest that this may be a general principle.

Matthews (203) has reported evidence for the existence of steps in the assembly of turnip yellow mosaic virus. By density gradient equilibrium experiments, he has obtained, in addition to intact virus containing 36 per cent RNA, non-infective particles with 24 per cent RNA, 12 per cent RNA, a "small amount" of RNA, and no RNA. Short-time studies of the rates of incorporation of ^{32}P and ^{35}S into these virus particles (obtained from the infected leaves) suggest a sequential relationship in which the protein shell is made first, after which RNA is inserted in several discrete stages.

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PROTEIN BIOSYNTHESIS[1,2]

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The discovery of enzymes that activate the amino acids and transfer amino acyl groups to RNA has provided a new model for the initial events of protein synthesis. Considerable work has been devoted to the study of these biochemical steps in order to prove or disprove their essentiality. Part of this review will refer to recent data in this field; the remainder will consider the large body of information presently available on the relationships between protein and gene structures, and on the regulatory mechanisms exerted at the level of the gene products (induction and repression of enzymes).

INTERMEDIARY STEPS IN PROTEIN BIOSYNTHESIS

Amino Acid Activation

Early observations by Hoagland, with soluble liver cell fractions (1, 2), that carboxyl groups of amino acids are enzymatically activated in the presence of ATP to form enzyme-bound amino-acyl-adenylates have been extensively confirmed with enzymes from other sources (3, 4). The continuously increasing list of highly purified systems that specifically activate single amino acids (5 to 9) leads to the generalization that each individual amino acid corresponds to its specific enzyme. A recent observation by Bergmann & Berg (10) constitutes an exception to this rule. Besides the existence, in *Escherichia coli* K 12, of an enzyme absolutely specific for valine, one finds an "isoleucine enzyme" that activates both L-valine and L-isoleucine. The constant ratio of the two activating properties at various stages of purification or inactivation proves that the system is not a mixture. L-Valine is activated at about 55 per cent of the rate of isoleucine, and the K_s for valine is approximately one hundred times higher than for isoleucine. No transfer of valine to $_s$RNA is mediated by the isoleucine enzyme, and valine competitively inhibits the transfer of isoleucine.

Aside from this very special case, if one considers carboxyl activation by ATP as the first biochemical step by which the amino acid residues are selected in protein formation, one must expect not only all the natural amino acids to be susceptible to activation, but also the amino acid analogues, which can substitute themselves for amino acids in a peptide chain. It has, in fact,

[1] The survey of the literature pertaining to this review was concluded in September, 1959.

[2] The following abbreviations are used in this review: AMP for adenosine monophosphate (adenylic acid); CMP for cytidine monophosphate (cytidylic acid); CTP for cytidine triphosphate; GTP for guanosine triphosphate; $_s$RNA for soluble ribonucleic acid; UTP for uridine triphosphate.

been observed with various systems that the L-isomers of amino acids can all be converted to amino-acyl-adenylates (11 to 13). Furthermore, the observation with analogues of tryptophan, that only those which are known to be incorporated into protein can be activated (14), has been extended to analogues of other amino acids, such as selenomethionine (15), norleucine (15), ethionine (15), and parafluorophenylalanine (15, 16), all of which can substitute for their natural counterparts and have been found to be incorporated (17 to 20). In contrast to these findings is the observation by Rendi *et al.* (21) that a purified methionine-activating system from rat liver does not activate ethionine, although this substance is incorporated into proteins. Activation of ethionine by cruder preparations of this tissue has, however, not been tested.

Curiously enough, the activating enzymes which appear to fulfill the right criteria for selecting amino acids residues, by the test of amino acyl adenylate formation, seem of little specificity in respect to their reverse action on amino-acyl-adenylates in the presence of pyrophosphate (22, 23). A yeast preparation which selectively activated L-methionine, as measured by the exchange reaction, not only converted the L-methionine adenylate but also the D-methionyl and even the L-seryl and L-phenylalanyl derivatives into the corresponding free amino acids. However, the pyrophosphorolysis of amino-acyl-adenylate is probably a reaction of little occurrence *in vivo* because of the low level of free pyrophosphate ions in the cell.

Transfer of Amino Acids to $_s$RNA

The mechanism of transfer of amino acids to $_s$RNA, a reaction discovered by Hoagland *et al.* (24, 25) and by Ogata *et al.* (26, 27), has been widely explored by many groups of workers during the last year.

Mechanism of amino acid binding to $_s$RNA.—It may be worthy of mention that free radioactive synthetic amino-acyl-adenylates can react with $_s$RNA and can label it (28, 29). The extent of such non-enzymatic labeling is small because of rapid decomposition of the synthetic anhydrides, but, if these compounds are mixed with purified activating enzymes, specific as to their amino acyl moiety, a considerable labeling is observed (30). According to the authors (30), stabilization of the mixed anhydride linkage by the enzyme would be the only means of permitting the transfer. But since the tryptophan-activating enzyme also binds and presumably stabilizes other amino-acyl-adenylates and does not transfer the amino acid group to the $_s$RNA, stabilization of the mixed anhydride linkages can only be a partial explanation for the "transferring" properties of the activating enzymes. Indubitably, specific catalytic properties must be attributed to these enzymes to explain their ability to carry a given amino acid in a precise way to the correct polynucleotide chain. However, the results of Wong *et al.* (30), confirming Berg's first observations (31), emphasize that no enzyme other than the specific activating enzyme is required for amino acid attachment.

This last conclusion is true only when the acceptor $_s$RNA has certain chemical features that allow it to react with activated amino acids. If the

$_s$RNA does not have these features, it has to be prepared by complex enzymatic events to accept amino acids. It had already been shown that, when preincubated with a pH 5.0 enzyme preparation plus pyrophosphate, $_s$RNA loses its ability to bind amino acid but recovers it if CTP and ATP are added to the mixture [Hecht *et al.* (32)]. Very careful analysis of this phenomenon has established that both CTP and ATP contribute, by their nucleotidyl fraction, to the reversible end-group labeling of $_s$RNA, and that the terminal nucleotide grouping thus obtained, RNA-CMP-CMP-AMP, is an obligatory feature for the attachment of all amino acids so far tested [Hecht *et al.* (32 to 34)]. Herbert (35, 36) has succeeded in purifying, by chromatography on an hydroxyapatite column, a single system, free of the amino acid activating property, that catalyzed the reversible and sequential transfer of both CMP and AMP. When a mixture of CTP and ATP was used as a substrate, CMP was added first, and AMP then covered the cytidyl residue. With this purified system, contrary to what had been observed with cruder preparations (33), the ratio of CMP to AMP incorporated was one instead of two. GTP and UTP are poor nucleotidyl donors, and they were not cofactors for amino acid attachment to $_s$RNA.

That the reaction of end-group addition of nucleotides to RNA is of general importance in cell economy is suggested by the previous work of Goldwasser (37), Heidelberger (38), and Canellakis (39) and also by recent observations that certain enzymes can transfer specifically the CMP residue from CTP to thymus RNA (40) and some others to DNA (41), $_s$RNA being an inactive acceptor for such systems. Having shown a requirement of terminal nucleotides for the amino acid attachment to $_s$RNA, it remains to be established that activated amino acids are, in fact, bound to these terminal units.

The observation by Zachau *et al.* (42) that the carboxyl group of amino acids esterifies the 2′ or 3′ free hydroxyl group of an adenosine residue in the $_s$RNA has been confirmed by Preiss *et al.* (43), who isolated as adenosyl amino acids the soluble fraction from ribonuclease digests of ^{14}C-leucyl- and ^{14}C-valyl-$_s$RNA complexes of *E. coli*. It has been definitively established by independent techniques that the adenosine residue involved in this kind of binding is the one in terminal position of the RNA-CMP-AMP sequence. In particular, the removal from the polynucleotide chain of the terminal unit bearing the unesterified 3′-hydroxyl group, by periodate oxidation followed by alkali treatment, entirely destroys the amino acid binding capacity of the $_s$RNA (43), as does a very limited treatment by snake venom phosphodiesterase, which splits RNA from the end of the chain (43), and which has been shown to liberate quantitatively the terminal CMP and AMP residues.

Specificity of the binding sites.—Although all the amino acids are probably attached to the same terminal nucleotide grouping, the piece of $_s$RNA exclusive of this grouping probably serves to determine which special amino acid is placed in the terminal position.

That preparations of „RNA in fact contain specific binding sites is shown by the additive and non-competitive labeling of „RNA from a mixture of radioactive amino acids (25, 44) and by the observation that a given amino acid, when bound to „RNA, protects selectively its binding site from periodate inactivation (34, 43).

Since the binding process is terminal and specific, it can be inferred that the cell contains a population of „RNA molecules, each of them specific for a given amino acid, rather than a single molecular species bearing as many sites as there are amino acids.

Such reasoning has led to fractionation of the crude „RNA, inasmuch as Schweet *et al.* (45) had provided evidence that certain chemical procedures can enrich „RNA in fractions that accept some amino acids more efficiently than some others. Although this problem is still in its infancy, successful isolation of pure specific „RNA molecules has been reported. By use of the countercurrent distribution method, Holley *et al.* (46) have considerably purified the specific acceptor for alanine, whereas, by the use of a special chromatographic device, the acceptor for tyrosine has been claimed to be also isolated (47).

Does a given „RNA molecule in accepting a given amino acid have the same structure among cells of different species? The answer to this question is conflicting. On the one hand, a valine-activating enzyme from rat liver has been reported to incorporate this amino acid as efficiently in „RNA from rat liver as in yeast RNA (34). On the other hand, it was observed recently (48) that a partially purified methionine-activating enzyme from *E. coli* incorporates three times as much methionine in the „RNA from *E. coli* as does purified activating enzyme from yeast. It would seem that the „RNA of *E. coli* contains methionine sites that the yeast enzyme cannot recognize, that is, two extra sites which are probably absent from the yeast „RNA.

Composition and properties of the „RNA.—„RNA from various sources contains atypical bases in high proportion. Among these additional components are pseudouridine (49 to 52), methylated purines (53 to 56), and 5-methyl-cytosine (57, 58). An atypical sugar has also been found (59). Only traces of these substances have been found in ribosomal RNA (60). The presence of thymine in the bulk yeast RNA has also been reported (53), but its relative proportion in the „RNA has not been studied.

Per 100 moles of nucleotides, the composition of „RNA from *E. coli* has been reported (61) to be: adenine 20.2 moles, uracil 20.1 (including 3.1 moles of uracil in an unidentified linkage), guanine 30.5, and cytosine 28.8 moles; whereas the composition of the inactive RNA is: adenine 25.5, uracil 20.9, guanine 31.2, and cytosine 22.3 moles. Not only is it clear that the two types of RNA differ considerably, but it may be of interest that the acceptor RNA shows a DNA type of base pairing. End-group analysis of the „RNA from *E. coli* (61) or rat liver (60) shows that 95 per cent of the end groups are adenosine, in agreement with the finding of Hecht *et al.* (33) reported above. In the

case of *E. coli* $_s$RNA, there is one such terminal adenosine for every 100 nucleotides, which suggests an average mol. wt. of $33{,}900 \pm 3400$ (61). Recent estimations based on other techniques give a value of 35,000 plus or minus 10 per cent for the *E. coli* $_s$RNA (62) and of 30,000 for the $_s$RNA of rat liver (25, 60). Recent work by Tissières has called attention to the existence of a secondary structure in the $_s$RNA (62) by showing that this polynucleotide exhibits a marked hypochromicity. It is not known, however, whether such a superstructure plays a role in the biological activity of the $_s$RNA.

Evidence for the Role of the Acceptor RNA in Protein Synthesis

$_s$RNA possesses the property of selecting amino acid residues ultimately found in peptide linkage because each natural amino acid carried by its specific activating enzyme can probably be recognized by a distinct specific $_s$RNA molecule. An illustration of this property is the observation that, in the $_s$RNA from guinea pig mammary cells, separate specific sites exist for glutamic acid and glutamine, which are known to be incorporated independently into milk protein (63).

Incidentally, it must be noted that incorporation of amino acid analogues as a tool for testing the potential selection exerted at the level of the $_s$RNA has not received much attention. It has only been observed that $_s$RNA from *E. coli* incorporates radioactive *p*-fluoro-phenylalanine when incubated with a pH 5.0 enzyme preparation (64). Incorporation of L-alloisoleucine-^{14}C and of L-norvaline-^{14}C by $_s$RNA from liver has been reported (34). It has been found that L-alloisoleucine-^{14}C is incorporated into $_s$RNA from rabbit reticulocytes, although the analogue is incorporated only at a small rate into protein (65). This observation suggests that partial rejection of an analogue can take place at a stage subsequent to its transfer to $_s$RNA.

The demonstration that attachment of amino acids to $_s$RNA is an intermediary step in protein formation had, however, to await experiments which showed that, *in vivo*, amino acids are incorporated into $_s$RNA prior to their incorporation into protein, and also that RNA-bound amino acids can be transferred directly into a peptide-like material.

Naturally-occurring $_s$RNA, when isolated by a procedure mild enough to respect covalent bonding with amino acids, contains almost all the natural amino acids attached to it (42, 66, 67). The behavior of these RNA-bound amino acids has been studied *in vivo* by Hoagland *et al.*, who showed that, when radioactive amino acids are injected into an animal, they label the $_s$RNA earlier than any other fraction of RNA or protein (25). Similar results have recently been obtained (68) during kinetic studies on amino acid incorporation into cells of *E. coli* during the exponential growth phase. The RNA amino acid pool (the pool of amino acids bound to the "total" RNA extracted from the cells) is saturated very quickly (5 min. at 20°C.). Inhibition of protein synthesis slows the rate of labeling of the pool, or the rate at which

unlabeling takes place by dilution of the tracer, without changing the final equilibrium. It is assumed that the availability of sites on transfer RNA is a direct function of the rate at which these sites are made available by subsequent steps in protein formation. Another interpretation is, however, possible: since the RNA-amino acid pool is in fact a mixture of the amino acids bound to $_s$RNA and of those bound to the particulate RNA, inhibition of protein synthesis may reduce the rate of labeling of the particulate RNA, while amino acids would gradually pile up in the $_s$RNA at a level above the normal value. This would indicate an absence of transfer of amino acids from the $_s$RNA to the particulate RNA. Studies on the labeling of separated RNA fractions are now in progress. Whatever the interpretation may be, it has been possible to show, under certain conditions, that up to 80 per cent of the radioactivity from the complex between RNA and a given amino acid can be transferred to protein without passing through a stage where it can be diluted with free non-radioactive amino acid. Similar conclusions have been reached by Gale (69) with disrupted *Staphylococci*.

Hoagland's first observations (25, 70) on systems, *in vitro*, probably serve better to illustrate that a direct transfer of $_s$RNA-bound amino acids to microsomal particles does, in fact, take place. It is worthwhile to recall that factors required for such transfer are GTP, ATP, and certain soluble enzymes.

More recently, on incubating $_s$RNA (labeled with ATP-^{14}C in its terminal AMP unit) with non-radioactive microsomes, Hoagland has found a labeling of the microsomal RNA and has calculated that 12 times as much adenine enters the microsomal RNA than does a single radioactive amino acid (70). Since $_s$RNA is known to contain unlabeled amino acids, all of which are attached to an adenine end, this result indicates that all terminally bound amino acids are simultaneously transferred to microsomes. A recent experiment by Acs points toward the same conclusion: a $_s$RNA preparation stripped of all its naturally attached amino acids by the action of dilute alkali and then labeled with a single amino acid could not transfer this bound amino acid to the microsomal fraction (71). The simultaneous transfer of bound amino acids suggests that these amino acids are really assembled in a new peptide chain during their incorporation in the microsome fraction and not simply added to the terminal part of already existing microsomal protein.

The roles of ATP and GTP in the transfer reaction still remain a mystery. Zillig *et al.* (72), by use of bacterial systems, have not detected any GTP requirement for the transfer to *E. coli* microsomes of $_s$RNA-bound amino acids. The soluble fraction that appears indispensable for the transfer to microsomes has been preliminarily purified from a pH 5.0 enzyme preparation (73). Vitamin B_{12} involvement as a cofactor in the overall reaction has been suggested (74).

Finally, although a certain radioactivity from $_s$RNA labeled with orotic acid (75) or ^{32}P (76) is found in the microsomal RNA at the end of incubation,

it is not known whether nucleotide fragments, exclusive of the terminal CMP or AMP units, accompany the amino acids during their transfer.

Other Pathways for Amino Acid Incorporation

Although the preceding results from experiments both *in vivo* and *in vitro* permit the amino acid activation process and the transfer to $_s$RNA to be considered intermediary steps in protein formation, they do not establish that such steps constitute an obligatory path.

A series of transfer experiments, *in vitro*, has suggested the existence of other pathways for amino acid incorporation into peptide linkages. A system that catalyzes the incorporation of radioactive amino acids into ribonucleoprotein particles and does not involve amino acid activation or, apparently, $_s$RNA has been obtained, by Beljanski & Ochoa from various sources (77, 78). This "amino acid incorporation enzyme" can replace the pH 5.0 enzyme system for stimulating amino acid incorporation into protein of rat liver microsomes. It has also been observed that particulate preparations of *Alcaligenes faecalis* are able to incorporate different ^{14}C amino acids into protein (79) and that they are free of known "amino acid activating enzymes" but contain endogenous "amino acid incorporation enzymes." Incorporation of valine-^{14}C, in the presence of a mixture of 18 L-amino acids, with preparations of particles solubilized in perfluorooctanoate takes place within a peptide chain. Recent studies indicate that the incorporation enzyme could possibly catalyze some new kind of amino acid activation, since it has been shown to contain four distinct Mg^{++}-dependent kinases that are able to exchange radioactivity from diphosphonucleotides with homologous corresponding triphosphonucleotides (80). It is not definitely known, however, whether these exchange reactions are required for the stimulation of amino acid incorporation.

Like the previous incorporation enzyme, the "S protein" is a fraction obtained by Sachs (81) from a pH 5.0 enzyme preparation, which contains apparently very little $_s$RNA and yet promotes active labeling from radioactive amino acids of microsomes. Glutathione is required for activity with the dialyzed preparation. It has to be stressed, however, that the question of whether or not classical activation systems are involved in the stimulatory properties of the "S" protein is still under debate. On one hand, it is stated by Rendi & Hultin (82) that amino acid activation by the classical exchange test does not appear to be mediated by the "S" protein (82); but on the other hand, a transfer of radioactive amino acids to $_s$RNA has been observed (82, 83), and it has also been reported that $_s$RNA promotes a certain stimulation of amino acid incorporation by particulate fractions in the presence of undialyzed "S" protein (82). One has to be cautious in giving definitive interpretations as to the possible occurrence of an alternate pathway, since only a small amount of exchange activity is necessary to catalyze incorporation on to RNA.

A series of observations from the various laboratories, made with microsomal preparations (84, 85), mitochondrial fractions (86, 87), or particulate components associated with the bacterial membrane (88 to 90), suggests that carboxyl activation of amino acids and formation of RNA-amino-acid intermediates may not be obligatory paths. Certain interesting features of amino acid incorporation have been observed with fractions obtained from protoplasts lysates: in a system composed of a mixture of ribosomes with a soluble fraction, called "amino acid polymerase," extracted from material sedimented at 30,000 g, an important incorporation of radioactive amino acids in a protein-like fraction was observed. This incorporation was stimulated by the addition of $_s$RNA to the mixture, even in the presence of ribonuclease, as if the nucleotides derived from $_s$RNA were involved in some process of amino acid activation (88, 89)—a result to be compared with the observation of Beljanski (80) reported above. It has to be stressed that failure to demonstrate the requirement for an intermediate does not constitute proof of its inessentiality, and that it is hard to exclude definitely the presence of an acceptor RNA in every system that has been used. Furthermore, the observations reported above show that, at least in one case, the S protein, the soluble system, has the property of forming RNA-amino acid complexes, suggesting that traces of exchange activities may be present in the system in sufficient amount to catalyze a transfer to $_s$RNA. It may very well be that there are, in fact, alternate pathways for protein synthesis. For instance, a possibility exists that structural protein of the ribosomes themselves are made by a mechanism differing from that involved in the formation of soluble proteins.

Metabolic Pools and the Synthesis of Protein

In *Candida utilis*, two operationally distinct amino acid pools are found (91). One accumulates amino acids within the cell to levels exceeding their concentration in the external medium; it has been called a "concentrating" or "expandable" pool and, in some of its properties, resembles the amino acid permeases found in *E. coli* (92). Its size varies with the external concentration, it is sensitive to osmotic shock and is readily exchangeable with external amino acids. The second pool is called the "internal" or "conversion" pool; in the absence of exogenous amino acids, this pool is formed solely from the carbon and energy source (glucose). The size of this internal pool is fixed during exponential growth; it is sensitive to osmotic shock, and its amino acids do not exchange with amino acids external to it. Once concentrated in the expandable pool, amino acids can provide material for the internal pool. If radioactive threonine is made available to an organism in its exponential phase of growth, more than 90 per cent of the radioactivity of the cold TCA fractions is contained in threonine, the remainder being in isoleucine, an amino acid that derives four of its carbons from the four carbons of threonine. However, it can be shown that all of this isoleucine and a

small quantity of the total pool threonine are components of the internal pool; these two components, as well as the threonine and isoleucine in the protein, have the same specific activity. Once radioactive threonine has entered the internal pool, if non-radioactive exogenous isoleucine is added, the threonine and the isoleucine found in the internal pool and in the protein still have the same specific activity. Similarly, as soon as aspartic acid-^{14}C has entered the internal pool, addition of members of the same "family" (i.e., amino acids that derive their carbons from aspartic acid, viz threonine, isoleucine, methionine), does not change the ratio of the specific activities of these amino acids in the internal pool or in the protein. Addition of isoleucine-^{12}C to a culture growing in fructose-^{14}C also does not result in a significant excretion of isoleucine-^{14}C. On the other hand, addition of aspartic acid-^{12}C causes a considerable excretion of aspartic acid-^{14}C (91).

These results are taken to indicate that there is no interconversion of amino acids in the expandable pool, which acts solely as a concentrating mechanism. Furthermore, at some point in the synthesis from fructose, family heads (aspartic, glutamic, and pyruvic acids) are formed and become available to the internal pool, where conversions to their respective family members occur. Once integrated in the internal pool, further competition of the amino acids with exogenous amino acids, expandable pool material, or with newly synthesized amino acids from fructose does not occur. The competition must take place prior to, or at the time of, incorporation into the internal pool. To satisfy this hypothesis, one has to visualize some structural arrangement of the enzyme leading from a family head to the derived amino acids which would allow the product of a given enzyme of the series to be passed onto the next enzyme as a substrate without ever being free or miscible with compounds outside the internal pool. One has also to visualize the fact that the amino acids are passed from this highly structured internal pool to the finished protein without mixing with exogenous amino acids. Although by the physicochemical properties of *E. coli* an internal pool of a size similar to that of *Candida utilis*, is not recognizable, experiments on isotope competition, similar to those described above, have given the same type of results.

Incorporation of Amino Acid Analogues

Numerous analogues of amino acids, when added to microbial cultures in the exponential growth phase, cause a transformation into a linear rate of growth. Concurrently, one or several enzymes cease to be synthesized in an active form and become limiting. It is tempting to think that this limitation is the cause of the linear growth. During this phase, proteins continue to be synthesized, as shown by incorporation of radioactive sulfur, radioactive amino acids, and by the presence of all the labeled amino acids in the proteins of bacteria grown in the presence of analogues with radioactive glucose as the sole carbon source (93). Two alternatives could explain these facts: (*a*) the analogue is incorporated *in lieu* of the corresponding natural amino

acid and gives rise to biologically active or inactive proteins; (*b*) the analogue inhibits incorporation of the natural amino acid and thus inhibits the synthesis of the proteins normally containing this amino acid. It has been shown that the first alternative accounts for the observed facts: *o*-, *m*-, and *p*-fluorophenylalanine, β-2-thienylalanine, norleucine (17), ethionine (20), selenomethionine (19), tryptazan (94), and 7-azatryptophan (95) are incorporated *in vivo* in the proteins of various microorganisms; norleucine was found to be incorporated in the casein of the lactating cow (96); and *p*-fluorophenylalanine in proteins formed in hen oviduct (97), in rat muscle aldolase and phosphoglyceraldehyde dehydrogenase (98), and in the hemoglobin from rabbit reticulocytes (99).

In one case, more thoroughly investigated (17), it was found that *p*-fluorophenylalanine stoichiometrically replaced phenylalanine in the protein synthesized by *E. coli* in the presence of the analogue. The extent of replacement can be considerable in microorganisms: 75 and 95 per cent of the phenylalanine with *p*-fluorophenylalanine and thienylalanine, respectively, and 65 per cent of the methionine with norleucine. These analogues do not affect in a significant manner the relative proportions of the amino acids which are not structurally related to them. It seems then likely that the incorporation of an analogue does not result in the synthesis of a radically different molecular species, but that the substitution by the analogue is the only change. This point has been further demonstrated in connection with norleucine substitution for methionine in the proteins of *E. coli:* by use of a methionine-requiring mutant and appropriate concentration of norleucine and radioactive methionine, a reduction of 38 per cent of the protein methionine was obtained (100). The bacterial proteins were separated into resolvable "protein classes" through the use of diethylaminoethyl-cellulose ion exchange columns. Characteristic regions were chosen on the elution pattern, corresponding to known enzymes, to well-resolved radioactivity and protein peaks, and to a ribonucleoprotein peak. The similarity of the elution diagrams obtained with labeled methionine or labeled norleucine showed that proteins formed in the presence of the analogue were physicochemically similar to the proteins normally synthesized. This finding also eliminates the suggestion that only certain proteins are susceptible to analogue substitution. The formation of incomplete proteins by the joining of the analogue by a peptide bond to one of its neighboring amino acids, but not to the other, does not seem to be probable. Should such unfinished molecules be present, they would markedly alter the elution patterns obtained after the analogue is incorporated. In addition, use of a methionine-requiring mutant to eliminate competitive reactions involving sulfur compounds other than methionine, and an external ratio of norleucine to labeled methionine such as to permit a 40 per cent substitution, resulted, on column resolution and isolation of the proteins from the seven best defined regions of the elution pattern, in an equal degree

of norleucine substitution throughout—approximately 40 per cent. Therefore, each methionine incorporation site seems to have an equal probability of analogue substitution (100).

Recently, crystalline α-amylase has been obtained from the culture medium of a methionine-requiring mutant of *Bacillus subtilis* grown in the presence of methionine plus ethionine. This α-amylase contained peptide-bound ethionine (101) (four moles of methionine, or methionine plus ethionine per mole of protein). After tryptic digestion, three of the four methionine-containing peptides were isolated; the degree of substitution in each peptide was 36 to 40 per cent, as compared to 39 per cent in the whole α-amylase molecule. These results (101) indicate that all four methionines have an equal chance of being replaced by ethionine and are in perfect agreement with the experiments described above (100).

Where is the selection made between the natural amino acid and the unnatural analogue? This question has been answered by an elegant experiment (102): two cultures of *Candida utilis* were grown in media containing, respectively, *p*-fluorophenylalanine-^{12}C + phenylalanine-^{14}C and *p*-fluorophenylalanine-^{14}C + phenylalanine-^{12}C, the ratio of the analogue to phenylalanine being 10. The cultures were allowed to increase to 20 times their original mass; during this period of growth, nine aliquots were taken and the ratios of radioactive analogue to radioactive phenylalanine were determined in the expandable pool, the internal pool, and the proteins. It was found that the ratio, analogue/phenylalanine, was totally unrelated to the ratio observed in the expandable pool or in the external medium. This result is taken to mean that the amino acids are transferred from the internal pool to the finished protein without the intervention of intermediate structures exhibiting a different selectivity factor toward the amino acid and the analogue.

Last Steps of Protein Synthesis

The probable existence of a secondary structure in $_sRNA$ and the presence of a "DNA type" of base pairing might suggest that $_sRNA$ is genetically determined. However, its low molecular weight and the binding of no more than a single amino acid per molecule do not favor the idea that the acceptor RNA is a template for peptide-bond formation. Therefore, the most reasonable assumption is the adaptor hypothesis suggested by Crick (103): the $_sRNA$ or a piece of this nucleic acid places amino acids in proper order at the surface of a template, which would possess the necessary features to code the sequence of the amino acids.

The current view is to consider ribonucleoprotein particles as sites of protein synthesis and, more specifically, as stable RNA templates containing the proper genetic information to code a linear arrangement of "free" or activated or nucleotide-bound amino acids. The most direct support for this view comes from a series of observations based on incorporation studies with

labeled amino acids, which show that, in cells or tissues synthesizing protein activity, radioactivity appears associated with microsomal ribonucleoprotein prior to detection in the soluble proteins [see Loftfield (104) for general discussion]. This has been established with animal cells (104 to 106) and also, more recently, with *Neurospora* (107) and bacteria (108). For instance, very short contact (about 10 sec.) of rapidly growing cells (logarithmic phase) of *E. coli* with ^{35}S-labeled sulfate led to an early appearance of radioactivity in the "ribosome" fraction, that is, the almost pure nucleoprotein stripped of the other usual protein and lipid contaminants of crude microsomal particles. This labeling preceded that of the soluble proteins. Repartition of the radioactivity after a pulse of the radioactive tracer showed that ribosomes accumulated a radioactive precursor, ultimately converted into soluble proteins when the external tracer was exhausted (108a). Ribonucleoprotein particles of *E. coli* exhibit various sizes (30 S, 50 S, 70 S, 100 S). Their shape, properties, composition, and metabolic interrelationships have been extensively studied (108, 109). Because of their heterogeneity of size, it is interesting to inquire into their respective activities in protein synthesis. All that preliminary studies seem to indicate is that the particles, the RNA and protein of which are labeled the fastest *in vivo*, are associated with the membrane (110).

Apart from these studies, *in vivo*, many interesting studies *in vitro* or mixed *in vivo-in vitro*, also point toward an active protein-synthesizing capacity for the ribonucleoprotein particles. Not only has it been shown that ribonucleoprotein particles (sometimes containing endogenous activating enzymes, ATP, and amino acids) can effect a net increase in protein-like material [Raacke (111)] or of a protein having enzymatic activity [Webster (112)], but it has been demonstrated, in some cases, that ribonucleoprotein particles can pile up precursors of specific proteins that are later secreted as finished proteins in the soluble phase. For instance, Rabinovitz & Olson (113, 114) have described a transfer of radioactivity from particulate sites to soluble hemoglobin by incubating ribonucleoprotein particles from labeled reticulocytes with unlabeled soluble fraction, ATP, Mg^{++}, and an ATP generating system. Also by use of particles of reticulocytes in incubation with the same type of supplements, including RNA, Schweet *et al.* (115) have shown that added radioactive amino acids are incorporated in the particles according to a ratio specific for the hemoglobin composition, and that the radioactive-like substance is secreted later as soluble hemoglobin. Results from Campbell *et al.* (116) also indicate that microsomes synthesize a protein material distinct from the microsomal protein fraction. After labeling *in vitro*, of rat liver microsomes and disintegration of the washed particles, they were able to separate, by precipitation with an antiserum, an albumin fraction considerably more labeled than the bulk of the other microsomal protein.

Certainly the best approach to the problem of the synthesis of a specific protein in subcellular fractions has been provided by the work of Simpson and his co-workers (116a) showing that a considerable amount of cytochrome-*c* can be synthesized by suspensions of mitochondria from liver; attempts are being made at present by these authors (116b) to fractionate the mitochondria in order to decide whether some special classes of RNP particles participate in this process of synthesis and also to establish whether amino acid-activating systems and $_s$RNA are involved in it.

Little is known about the mechanism that effects detachment of the finished protein from the particle, but Simkin (117) has recently shed some light on this process by studying how radioactive antibodies, synthesized *de novo* in the microsomes of spleen from immunized rabbits, are released in the free form. They have shown that the process definitely requires ATP and magnesium, together with the unlabeled cell sap of the spleen extract.

Although the sum of the observations reported indicates that the template for protein synthesis appears to reside in the metabolically stable RNA fraction of microsomes, recent kinetic studies on enzyme synthesis in bacterial zygotes are in interesting conflict with this hypothesis. As observed by Pardee, Jacob & Monod (118), when the piece of chromosome carrying the character Lac^+ is transferred from an Hfr strain to the cytoplasm of an F^- recipient strain which is genetically unable to make β-galactosidase (Lac^-), the synthesis of this enzyme starts almost immediately in the zygote. This gives no positive indication that a stable cytoplasmic replica of the gene must be accumulated prior to the formation of the new protein species. Also significant is the fact that, in zygotes obtained with a donor strain labeled in its DNA by highly radioactive ^{32}P, the enzyme-forming capacity decays at the same rate as the genetically active material, thus suggesting that gene integrity is required for enzyme synthesis (119). These results could be interpreted in three ways: (*a*) DNA serves as the direct template for protein synthesis; (*b*) some RNA particles accompany the chromosome during injection; these particles would function as templates for the enzymes, but only when the integrity of the chromosome to which they are attached is preserved; (*c*) there is formation in the cytoplasm of an RNA template with the proper information, but this RNA is so unstable that formation of the specific protein requires the continuous presence of the gene.

At present, it seems impossible to postulate the nature of this hypothetically unstable RNA which is genetically controlled, except that it bears some resemblance to the highly unstable and metabolically active RNA fraction which is synthesized immediately after phage infection (120, 121).

It must be noted that the need for the continuous presence of the gene to make enzymes in zygotes (119) [and also, as already described, in bacteria (122)] is in conflict with the results of enucleation experiments in cells of

higher organisms (123, 124) and with the behavior of naturally occurring enucleated cells such as reticulocytes (125, 125a). These cells, it is true, may represent an extreme case, since they are highly specialized in the formation of a single protein entity.

MUTATIONAL ALTERATIONS IN PROTEINS

In the original one gene-one enzyme theory, it was believed that mutation of one gene caused the suppression of the synthesis of one enzyme. In an increasing number of cases, mutation has been shown to cause the synthesis of a protein sufficiently altered to cause the loss of enzyme activity, but retaining some properties of the unaltered enzyme, such as antigenic structure or affinity for the substrate. Such cases have been reported for *Neurospora* tyrosinase (126), pantothenic acid synthetase (127), and tryptophan synthetase (128). In one case, that of human sickle-cell hemoglobin, the protein has been shown to differ from the normal form by a simple amino acid substitution (129). This last case strengthens the hypothesis that the linear amino acid sequence of each protein may reflect the linear sequence of the nucleotides of its determining gene (103). Other mutations apparently lead to other single amino acid changes in hemoglobin (130); unfortunately, our meager knowledge of human genetics does not allow the establishment of a linear relationship between DNA and protein structures.

Tryptophan synthetase catalyzes the three following reactions (131):

$$\text{Indole} + \text{L-serine} \rightarrow \text{L-tryptophan} \qquad 1.$$

$$\text{Indole-glycerolphosphate} \rightleftharpoons \text{indole} + \text{triosephosphate} \qquad 2.$$

$$\text{Indole-glycerolphosphate} \rightarrow \text{L-tryptophan} + \text{triosephosphate} + \text{L-serine} \qquad 3.$$

Reaction 3 appears to be normally responsible, *in vivo*, for growth in the absence of added tryptophan. The activity of the enzyme results from the cooperation of two distinct and separable protein species, A and B (132), the synthesis of which is controlled by two neighboring, but separate, genes (133). Both components must be present for appreciable progress of any of the three reactions. Mutations may cause altered forms of both components, which, added to the normal second component, can catalyze only one of the three reactions (134). It thus appears that enzymatic activity may be associated with the joint presence of separate polypeptide chains; this reminds one of the behavior of ribonuclease, which is active whether two separate polypeptide fractions are joined by a peptide linkage or not (135). It has been shown that a suppressor mutation causes the synthesis of an A protein with normal catalytic activity as well as the continued synthesis of the altered A protein characteristic of the unsuppressed mutant (136).

There have also been reports of independent mutations that cause the synthesis of an altered *E. coli* β-galactosidase, with all of the mutation sites

located within a single cistron, called the z cistron (136). It is possible to map them within this cistron, but the present knowledge of β-galactosidase structure does not permit us to decide whether each of these mutations causes a single amino acid change. The altered proteins are synthesized to an extent comparable to that of β-galactosidase itself, in wild-type induced cells. Four of these proteins give a complete cross-reaction with anti-β-galactosidase serum, whereas four others exhibit only a partial cross-reaction. In each class, one of the altered proteins has a weak galactosidase activity. These results show that the gene z contains information relative to the structure of β-galactosidase; experiments with z^+/z^- heterogenotes show that both the enzyme and its altered form are produced simultaneously; the cross-reacting material is thus a final product of the gene z^- and not an unfinished precursor of the enzyme. In addition, the cross-reacting materials, although having lost their enzymatic activity, have retained their properties of inducibility and repressibility (136).

The following facts lead us to believe that the gene z not only contains structural information for β-galactosidase, but the totality of this information. By transduction, the *lac* segment of *E. coli* has been transferred to *Shigella dysenteriae*, a species unable to synthesize β-galactosidase and the corresponding permease (137) (transduction is known to carry extremely small genetic segments). By recombination, the same segment has been transferred from *E. coli* to *Salmonella typhimurium* (138), which differs from the genus *Escherichia* in many bacteriological, biochemical, and antigenic characteristics. In both cases, the β-galactosidase produced was indistinguishable by its affinity, activity, and physical and immunological properties from that of the donor *E. coli* strain. It is clear that, if the structure of this protein were determined by genes other than the z gene or by some other genetic or biochemical "environment," such results would not have been expected.

NON-GENETIC ALTERATION OF PROTEIN STRUCTURE

The possibility that alterations in RNA structure or functioning could lead to the formation of altered proteins has already been suggested by Chantrenne (139) from the results of experiments with base analogues in bacteria. It had already been observed that 5-hydroxyuridine (139a) and 5-fluorouracil (139b, 139c) selectively inhibit the constitutive synthesis of β-galactosidase without preventing an increase in protein mass. Similarly, 8-azaguanine prevents the constitutive synthesis of penicillinase in *Bacillus cereus* (139).

It has actually been observed recently that *E. coli* grown in the presence of 5-fluorouracil manufactures altered enzymes. Two classes of alterations have been obtained: alkaline phosphatase with normal specific activity is formed, but this enzyme is less thermostable than the normal enzyme (140); on the other hand, addition of 5-fluorouracil leads to the formation of a pro-

tein antigenically related to β-galactosidase, but enzymatically inactive (141). Hamers (142) has independently reported that thiouracil-grown *E. coli* form, after induction, or constitutively, an enzymatically inactive protein which cross-reacts with β-galactosidase. Preliminary studies on amino acid incorporation in 5-fluorouracil-grown *E. coli* indicate that the bulk of the protein synthesized in the presence of the analogue contains less proline and tyrosine, but more arginine, than in the case of normal *E. coli*. It may be that the observed alterations of bacterial enzymes caused by the analogue are correlated with the changes in amino acid composition. Kinetic studies on the synthesis of the altered enzymes and on incorporation of proline and tyrosine in RNA suggest that the influence of the analogue on enzymes might be caused by a change in the structure of the adaptor RNA.

THE CONTROL OF ENZYME SYNTHESIS, INDUCTION, AND REPRESSION

Repression has been defined as the specific inhibition of the biosynthesis of enzymes, responsible for the synthesis of a given essential metabolite, by that metabolite, called the repressor (143). Excellent reviews have appeared on repression and the reader is referred to them (143 to 146).

Repression, exerted by the more or less direct products of the activity of an enzyme, causes the inhibition of its synthesis; while induction, exerted most often by a substrate of the enzyme, causes a rather considerable increase in its differential rate of synthesis. Under these conditions, it is reasonable to make the hypothesis that these two effects correspond to the same fundamental mechanism common to inducible and constitutive systems. This hypothesis can be expressed in two alternative forms: (*a*) The primary phenomenon is induction, in which case the repressor would act always as an antagonist of an exogenous or endogenous inducer (147). (*b*) The primary mechanism of regulation is negative, i.e., repressive, with the inducer functioning as an antagonist of an endogenous repressor (118, 143, 145, 148).

To confirm one of these two hypotheses, it was important to show either the intervention of an inducer in a repressible system, or the intervention of a specific repressor in an inducible system. The system described above, *E. coli* β-galactosidase, has provided an answer to this question. In addition to *z*, which has been defined as a structural gene, the inducibility or constitutivity of β-galactosidase (and of galactoside-permease) is under the control of i, a regulatory gene. The synthesis of the permease is under the control of a gene y. Each gene can exist in two forms y^+ and y^-, z^+ and z^-, and i^+ (inducible) and i^- (constitutive). The i gene is hypostatic to the two others. The three types of mutations are independent (149). The genetic analysis of these mutations shows that they affect a very small segment of the *E. coli* linkage group, that is, of its chromosome.

When it can be said that in wild-types z^+ i^+, the structural information is

delivered only in the presence of an inducer, and that, in z^+i^- mutants, it is spontaneously delivered, we shall be able to describe the experimental evidence for the repressive mechanism of regulation. The experiments consist essentially in the partial genetic transfer of a chromosome from a male cell to a female cell, the transfer being obtained by recombination (118), transduction (150), or by co-transfer with infectious F^+ factor (151). Whichever mode of conjugation is used, one obtains a meroheterozygote consisting of the female cytoplasm, two or three chromosomes from the female, and a chromosome segment from the male. The transfer concerns only the genetic material (DNA) from the male with exclusion of male cytoplasm; there is no cell fusion, and there seems to be no cytoplasmic injection. Two such transfers are made: Male z^+i^+ genome is introduced into female z^-i^- cytoplasm in the absence of inducer (none of the participants can synthesize enzyme under these conditions); as soon as the male material has entered, constitutive synthesis takes place and the rate of enzyme synthesis corresponds, if one takes into account the number of zygotes formed, to what would be expected if the z^+ gene acquired immediately its capacity to be expressed. After an hour or so, enzyme synthesis ceases, and the zygotes are now inducible. This is taken to mean that there is an immediate and complete interaction between the z^+ structure carried by the male chromosome and the i^- structure carried by the female chromosome. The important and necessary control is the reciprocal and symmetric cross; when a male z^-i^- genome is introduced into a female z^+i^+ cytoplasm, the genetic structure of the resulting zygote is identical with that of the inverse cross. The result is striking: no trace of enzyme is made, even after several hours. The only possible explanation of the opposed properties of the two types of zygotes is that the inducible or constitutive properties of the z^+ gene depend on the nature of its cytoplasmic environment, not on its immediate association with the inducible or constitutive form of the i gene. Several models could be given for such a result, and they have been discussed at length (146). Suffice it to say here that the most plausible is that the i^+ gene controls the synthesis of a specific repressor substance, the effect of which is nullified by the addition of inducer. In the i^- bacteria, this repressor is not synthesized.

An entirely parallel case has been found in a study of tryptophan biosynthesis by *E. coli* (148). Tryptophan represses the synthesis of the enzymes of its own biosynthetic pathway. Mutants have been found where this repression effect is abolished, which causes a profound increase in the synthesis of the enzymes concerned. These mutations affect a gene R Try distinct from those which control the capacity to synthesize these enzymes. The "depressed" allele (R^- Try) is recessive to the repressed one (R^+ Try). As the mutation does not affect the size of the tryptophan pool, it is clear that tryptophan itself is not the repressor; the latter must be synthesized from tryptophan, under the control of the R^+ Try gene (148). "Derepressed"

mutants have been obtained for methionine, valine, and arginine (148, 152).

It already appears from these observations and others under current study that most inducible systems and, perhaps, most biosynthetic systems in bacteria are under the control of a negative regulation, caused by a specific repressor synthesized under the influence of a specific regulatory gene. There is some evidence that protein synthesis is not necessary for the synthesis of the repressor (153, 154), which might thus appear as a direct product of the gene itself. The question of the chemical nature of the repressor is thus posed in a precise fashion. In so far as the mode of action of the repressors is concerned, several hypotheses have been formulated (146, 156), each of which leads to precise genetic predictions which are experimentally testable (155).

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BIOCHEMISTRY OF THE PROTEIN HORMONES[1,2]

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INTRODUCTION

The twenty protein hormones that have been studied to date constitute a very small family when compared to the 700 or 800 enzymes, which have been described. The question arises as to whether this discrepancy is attributable to the fact that nature seldom makes use of protein hormones or that they are difficult to uncover. However, although these hormones are similar in that they must all pass through the membrane of the cell that produces them and travel through the blood stream to reach their target cell, they are quite different in chemical composition and size. Certain of these hormones are among the smallest known biologically active peptides and as such have received intensive study; the complete amino acid sequence of seven of these hormones has been determined, and, to date, three have been synthesized. Actually, the protein hormone field has offered good preliminary training for more ambitious studies in the chemical structure of proteins.

From a chemical point of view, the present interest in the study of protein hormones is the same as that in general protein chemistry, that is, the relationship between structure and function or the explanation of biological activity in chemical terms. Two lines of attack are usually employed in the study of these problems: (*a*) chemical or enzymological modifications of the molecule, including the synthesis of analogues, to determine the active groups or the active sites; (*b*) comparative chemistry of the same functional molecule from different species; the natural substitution of some residues can show the relative importance of the nature or the position of amino acids in the peptide chain. Incidentally, the newest preparative methods now allow the purification of human hormones, a development which may eventually be of use in the medical field.

From a physiological point of view, it appears that the pituitary gland, for a long time assumed to be an autonomous director of the endocrine system, can be under neurohumoral control, some neurosecretory cells being able to produce hormones [Scharrer & Scharrer (1)]. The organization of the endocrine system has now to be considered at three levels with feedback control mechanisms. The central nervous system can secrete neural hormones, which stimulate the adenohypophysis; the adenohypophysis produces hormones that stimulate gonads, thyroid, adrenal cortex, etc.; the latter glands

[1] The present review covers the period from July, 1957, to July, 1959.

[2] The following subjects have been regretfully omitted: hypertensin, relaxin, thyroxine and thyroglobulin, parathormone, and gastrointestinal hormones.

make hormones which, besides their specific hormonal action, exercise a control over the central nervous system [Harris (2)].

NEUROHYPOPHYSEAL HORMONES[3]

Oxytocin and Vasopressin

Isolation.—Modern techniques of fractionation, i.e. zone electrophoresis, chromatography, etc., have permitted considerable simplification in the process of isolation. Column electrophoresis has been particularly utilized by the Swedish scientists [Porath (3); Kiessling & Elmqvist (4)]. Porath (3) has obtained from pig pituitaries highly purified oxytocin and vasopressin by the following method of purification: The acetic acid extract of post-pituitary powder is first fractionated with acetone and ether; the result is an active fraction containing 50 per cent of the initial oxytocin and vasopressin activity (avian depressor activity, 7 U.S.P. units/mg.; pressor activity, 7 U.S.P. units/mg.); the fraction is then treated in a differential manner, depending on which of the hormones is to be recovered. Oxytocin is obtained by a combination of one chromatographic step and one electrophoresis. The chromatography is performed on a column of triethylamino-cellulose. The product obtained (avian depressor activity, 20 U.S.P. units/mg.; pressor activity 19 U.S.P. units/mg.) is submitted to electrophoresis on cellulose columns 2.5 to 3 m. high with volatile buffer (formic acid-triethylamine or formic acid-pyridine). At pH 2.35, nine constituents can be separated after five days of electrophoresis. The oxytocin thus isolated is highly purified (avian depressor activity, 400 to 450 U.S.P. units/mg.), but, the vasopressin is contaminated with other substances and must be obtained through another method. This method consists of fractionation of the precipitate obtained with acetone-ether by two chromatographic steps and an electrophoresis. This precipitate is first passed through a column of diethylaminoethyl-cellulose, which adsorbs oxytocin and other impurities. Then the unadsorbed material is put onto a column of sulfomethyl-cellulose. Vasopressin is eluted with 80 per cent acetic acid, and the eluate (pressor activity 67 U.S.P. units/mg.) is submitted to electrophoresis. The lysine-vasopressin is isolated in a highly purified state (pressor activity 280 U.S.P. units/mg.). However, the yield of these two hormones has not been calculated.

The use of ion exchangers has permitted very rapid isolation of pure hormones [Ward & Guillemin (5); Schally *et al.* (6); Light *et al.* (7); Acher *et al.* (8)]. Starting with commercial extracts of beef and hog post-pituitaries (Protopituitrin), Ward & Guillemin (5) have obtained highly purified lysine-

[3] The cytological distinction between neurohypophysis (nervous tissue) and adenohypophysis (glandular tissue), which is reflected in the chemical similarity of certain hormones produced by these tissues (oxytocin and vasopressin as example of the former and corticotropin and intermedin for the latter), seems to justify a classification of the pituitary hormones into neurohypophyseal and adenohypophyseal hormones.

vasopressin (260 U.S.P. units/mg.) and arginine-vasopressin (380 U.S.P. units/mg.) by use of chromatography on carboxymethyl-cellulose. A gradient of pH and of ionic strength was produced with buffers of ammonium acetate. Schally *et al.* (6) have separated oxytocin and vasopressin by a similar method starting from Protopituitrin. The first hormone eluted, oxytocin, is passed through a second column of carboxymethyl-cellulose. The purity of these preparations was not studied. Acher, Light & du Vigneaud (8) have reported a rapid method for the simultaneous purification of the two hormones, which is based on the specific adsorption of oxytocin and vasopressin onto a protein of the post-hypophysis, neurophysin [Acher (9)]. As a first step, the oxytocin-vasopressin-neurophysin complex is precipitated with NaCl from an acid extract of post-pituitary powder, then purified by fractional precipitation at pH 3.9. In the next step, the protein-carrier is eliminated by precipitation with 5 per cent trichloroacetic acid, the peptide hormones remaining in the supernatant. Finally, oxytocin and vasopressin are purified by a single chromatographic separation on Amberlite IRC-50 (XE-64) with a pH and ionic strength gradient made with ammonium acetate buffers. The procedure was applied in the isolation of both oxytocin and vasopressin from man [Light & du Vigneaud (10)], horse, sheep, and chicken [Acher, Chauvet & Lenci (11 to 14); Chauvet *et al.* (14a)]. These hormone preparations are pure as tested by chromatography and paper electrophoresis and have specific activities of 400 to 500 U.S.P. units/mg. The overall yields, which vary from one species to another, are generally high, reaching 60 per cent in the case of sheep.

To the present time, the results obtained indicate that the same oxytocin exists in man, beef, hog, horse, sheep, and chicken. Arginine-vasopressin exists in man, beef, horse, and sheep [Light & du Vigneaud (10); Acher *et al.* (11, 14)]. On the basis of certain chromatographic [Heller & Lederis (15); Sachs & Barrett (16)] and pharmacological [Van Dyke *et al.* (17)] data, the vasopressin of rat, dog, monkey, and camel would seem to be arginine-vasopressin. The same neurohypophyseal hormones are found in the mammals thus far examined, with the single exception of hog which contains lysine-vasopressin instead of arginine-vasopressin [Popenoe *et al.* (18)].

Structure-activity relationships.—Oxytocin and vasopressin have very similar chemical structures and also possess the same biological activities, oxytocic and pressor, but to very different degrees. The synthesis of these hormones and also of "hybrids" and analogues has made possible, to a certain extent, a determination of the role of certain amino acid residues in the biological activity of the hormone molecules. Bodanszky & du Vigneaud (19) have elaborated a new and elegant synthesis of oxytocin, starting with the C-terminal amino acid and adding on successively the eight other residues by use of the acylamino acid nitrophenyl esters. Each addition is performed with a high yield (90 to 100 per cent), and no racemization is obtained. The elimination of the protecting groups from the final nonapeptide is done with sodium in liquid ammonia, with a 78 per cent yield.

Du Vigneaud and collaborators (20 to 23) have also improved their methods of synthesis of vasopressin. Among the several synthetic procedures employed, the best results were obtained for lysine-vasopressin by coupling the pentapeptide S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutamyl-L-asparagine with the tetrapeptide S-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysyl-glycinamide [du Vigneaud *et al.* (20)] and for arginine-vasopressin by coupling the azide of the tripeptide S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine with the hexapeptide L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginyl-glycinamide [du Vigneaud *et al.* (23)]. The synthetic and natural products have the same behavior in chromatography, countercurrent distribution, and electrophoresis and possess the same degree of biological activity.

The Cornell group has synthetized various analogues of oxytocin [Ressler & du Vigneaud (24); Ressler & Rachele (25); Lutz *et al.* (26); Bodanszky & du Vigneaud (27)] and of vasopressin [Katsoyannis & du Vigneaud (28)] and "hybrids" of the two hormones [Katsoyannis (29); Katsoyannis & du Vigneaud (30)]. Boissonnas and collaborators (31, 32, 33) and Rudinger *et al.* (34) have also synthesized analogues of oxytocin. The number and the diversity of the substances synthesized have led to confusion in the nomenclature, but it is possible to classify them into a single chemical family, that of oxytocin, by simply indicating in the prefix the nature and the position of the substituted residues.[4] Table I presents the different substances and their biological activities.

As can be seen from Table I, the substitution of a single residue in the oxytocin molecule, especially in the ring, is sufficient to diminish strongly the oxytocic activity. The replacement of the phenol group of tyrosine by a phenyl group (Phe^2-oxytocin) and the replacement of the branched group of isoleucine by a phenyl group (Phe^3-oxytocin) or by an isopropyl group (replacement of isoleucine by valine: Val^3-oxytocin) is accompanied by a decrease of activity. On the other hand, the presence of a strong basic group in the eighth position is necessary for the appearance of the pressor activity (Arg^8-oxytocin) and the pressor activity increases as the oxytocic activity is decreased by a substitution in the ring (Phe^3-Arg^8-oxytocin and Phe^3-Lys^8-oxytocin).

Besides its action on the uterus, oxytocin also has other strong biological activities, such as avian depressor activity and milk-ejecting activity. These activities are not similarly affected by the various substitutions; for example, Val^3-oxytocin is less active on the uterus but more active on the mammary glands than is oxytocin itself [Berde *et al.* (35)].

When the oxytocin molecule is shortened to a pentapeptide amide [Ressler (36)] or is lengthened to a nonapeptide amide [Guttmann *et al.* (37);

[4] The abbreviations of the amino acids residues are those of Brand, E., and Edsall, J. T. [*Ann. Rev. Biochem.* **16**, 224 (1947)] and the sequential arrangements are patterned after those suggested by Sanger, F. [*Advances Protein Chem.*, **7**, 1 (1952)].

TABLE I

THE CHEMICAL FAMILY OF OXYTOCINS

1 2 3 4 5 6 7 8 9
CyS·Tyr·Ileu·Glu(NH_2)·Asp(NH_2)·CyS·Pro·Leu·Gly(NH_2)

Analogue	*Other name*	*Oxytocic activity**	*Pressor activity**
Oxytocin		500	7
Phe^2-oxytocin		31	—
Val^3-oxytocin	Valyl-oxytocin	(170)	—
Leu^3-oxytocin		(15)	—
Phe^3-oxytocin	Oxypressin	20	3
IGlu(NH_2)^4-oxytocin	Isoglutamine-oxytocin	0.01	0
IAsp(NH_2)^5-oxytocin	Isoasparagine-oxytocin	0	0
Glu(NH_2)^5-oxytocin		—	—
Arg^8-oxytocin	Arginine-vasotocin	75	125
Phe^3-Arg^8-oxytocin	Arginine-vasopressin	30	600
Phe^3-Lys^8-oxytocin	Lysine-vasopressin	5	300
Phe^3-His^8-oxytocin	Histidine-vasopressin	1.5	1.5

* U.S.P. units per mg.

Konzett (38)], the oxytocic activity is lost. Certain analogues and homologues not only show no biological activity, but also inhibit the action of oxytocin and vasopressin. Isoglutamine-4-oxytocin is an inhibitor of vasopressin [Ressler & Rachele (25)], and the oxytocin homologue that has one supplementary tyrosyl residue is, in high concentrations, an inhibitor of oxytocin [Guttmann *et al.* (37)].

Important information concerning the groups and the bonds required for biological activity can be obtained by chemical or enzymological modifications of the natural hormones. Several investigators have studied the effect of reduction of the disulfide bridge [Dicker & Greenbaum (39); Audrain & Clauser (40)]. Dicker & Greenbaum (39) incubated Pitressin (commercial vasopressin) with cysteine or glutathione and found that, while the pressor activity remained unchanged, the antidiuretic activity was diminished by a third. On the other hand, Audrain & Clauser (40) treated Pitocin (commercial oxytocin) with cysteine and then determined the uterus activity under anaerobic conditions. They observed that the oxytocic activity was decreased by about 80 per cent. Reoxidation by air or H_2O_2 brought back most of the original activity. These authors concluded that the reduced hormone is itself inactive. When the classical assay was performed under aerobic conditions, however, reoxidation was spontaneous; it can be claimed, therefore, that both forms, oxidized and reduced, are active.

Ressler (41) has studied the formation of a dimer of oxytocin. When a sample of the hormone (350 units/mg.) was allowed to stand in a solution of $NaHCO_3$ at pH 8.3 at room temperature, a white product precipitated with a yield of 40 per cent. This product exhibited physical properties (optical rotation, solubility, electrophoretic mobility) different from those of oxytocin and possessed a very weak biological activity (7 units/mg.). The molecular weight determined by osmometry was 2250. The reduction of the dimer by cysteine, homocysteine, or glutathione yielded a product with an activity of about 200 units/mg., probably by reforming the monomer. Ressler (41) has also suggested the existence of denaturated peptides. This very interesting observation resulted from dissolving oxytocin in 7 *M* urea and following, as a function of time at pH 8 and 4.3, the variations of optical rotation and of biological activity. There was a parallelism between the increase of levorotation and inactivation. Treatment with cysteine did not reactivate the hormone. Certain secondary bonds, perhaps of hydrogen-bond character, would therefore seem to be important for the activity of the molecule.

Biological effects: metabolism.—Thorn (42) and Taubert (43) have published very pertinent reviews on the physiological and clinical effects of vasopressin. Important contributions in this field were reported during a symposium on the neurohypophysis [Heller (44)].

An assay of oxytocic activity based upon the induction of labor in the pregnant rabbit was proposed by Berde & Cerletti (45). Also, biological assays of vasopressin utilizing its antidiuretic action [Andersson & Persson (46); Dettelbach & Sweeney (47)] or increase in kidney weight [Binkley & Christensen (48)] have been described. Qualitative differences that limit the accuracy of quantitative comparisons between the two hormones have been demonstrated between the antidiuretic action of arginine- and lysine-vasopressin [Sawyer (49); Nielsen (50)]. Lysine-vasopressin (hog vasopressin) is less active than arginine-vasopressin in the dog, but it is as active, if not more so, in the hog [Munsick *et al.* (51)].

The biosynthesis of oxytocin and vasopressin has been little studied. These hormones are formed in the hypothalamus, but in different sites [Chamorro & Minz (52 to 54); Duggan & Reed (55)]. Several investigations have been made on the presence in the blood of substances having oxytocic activity [Reuse & Van den Driessche (56); Hawker & Robertson (57)], as well as their destruction by specific enzymes [Tuppy & Nesvadba (58); Semm (59)]. Tuppy & Nesvadba (58) have demonstrated in the blood of pregnant women, an "oxytocinase" with the specificity of an aminopeptidase; the enzyme exhibits optimal activity at pH 7.2 to 7.7 and is capable of hydrolyzing amino acid β-naphthylamides, in particular L-cystine-di-β-naphthylamide. The existence in the urine of substances that have oxytocic [Waleszek (60); Aonuma *et al.* (61, 62); Gomes (63)] and antidiuretic [Bellens (64); Bellens *et al.* (65)] activity has been observed, but their identity with the neurohypophyseal hormones remains to be proven.

The mode of action of oxytocin and vasopressin is still unknown. Ginet-

zinsky (66) has reported that the antidiuretic hormone favors the secretion by renal cells of a hyaluronidase that depolymerizes the intercellular cement, thereby permitting the reabsorption of water. The influence of vasopressin on the transport of electrolytes across biological membranes has been explored [Morel *et al.* (67); Brooks & Pickford (68); Thorn & Milewski (69)].

ADENOHYPOPHYSEAL HORMONES

The Intermedins

Isolation of α and β intermedins.—Li (70) has written a very detailed review of the hormones that act on melanocytes, called intermedins, melanotropins, or melanocyte-stimulating hormones (MSH). These hormones are of two chemical types: the type α, characterized by blocked N- and C-terminal groups, and the type β characterized by free N- and C-terminal groups. To date, the type α has been found only in the hog, whereas the type β has been found in hog, beef, and man [Harris & Lerner (71); Harris (72, 75); Harris & Roos (73, 74); Geschwind & Li (76); Geschwind *et al.* (77, 78)].

The separation of the two intermedins of pig was effected by electrophoresis and chromatography [Lee (79); Steelman *et al.* (80)]. After extraction of hog post-pituitary powder by glacial acetic acid, precipitation by ether, and adsorption on oxycellulose, Lee (79) separated the active substances by starch electrophoresis at pH 4.55 and 8.9. At pH 8.9, the α-hormone is displaced toward the cathode and the β-hormone toward the anode. These two substances are present in acetone powders as well as in lyophilized glands. Steelman *et al.* (80) have proposed a simplified procedure for the preparation of the two hormones, applicable to large quantities of material. The authors start with a by-product of the preparation of corticotropin. The corticotropin in the extract of post-pituitary is first removed by adsorption onto a very small quantity of oxycellulose, and then the two peptides desired are fixed by an excess of the absorbant and recovered by elution with HCl. This solution is lyophilized; the product, redissolved in water and dialyzed, is then chromatographed on a column of carboxymethyl-cellulose equilibrated with 0.01 *M* ammonium acetate at pH 5.8. The column is washed with the buffer. Under these conditions, the β-hormone (isoelectric point, pH 5.2 to 5.8) is not retained, but the α-component (isoelectric point, pH 10.5 to 11) is fixed and can be eluted in turn by use of a gradient of pH and ionic strength made with 0.1 *M* ammonium acetate at pH 6.5. Each product is chromatographed twice on carboxymethyl-cellulose, the β-hormone with 0.01 *M* ammonium acetate at pH 5.0 and the α-hormone with a gradient of this same buffer from 0.01 *M*, pH 5.5 to 0.1 *M*, pH 6.5. The products were compared with the pure hormones isolated by other procedures. The α-melanocyte-stimulating hormone was slightly contaminated with lysine-vasopressin, but the latter could be easily removed by treatment with thioglycollic acid, which reacts with lysine-vasopressin to give an acid derivative, and subsequent separation by chromatography or ionophoresis. The amino acid composition of the final product corresponded to that determined by Harris & Lerner (71) for the

pure hormone, and its activity was of the order of 1000 I.U./mg.[5] The β-hormone possessed an electrophoretic mobility and a biological activity identical with that of the pure hormone isolated by Geschwind & Li (76). Quantities of the order of 10 to 20 mg. of the hormones can be prepared by this method, but the yields are not indicated. The β-hormone has also been obtained by countercurrent distribution [Geschwind *et al.* (78); Sato & Katano (81)], and human β-hormone has been prepared by use of ion-exchangers [Dixon (82)].

Structure-activity relationships.—As a result of the investigations of Harris, Li, and their collaborators on amino acid sequence and those of Boissonnas, Hofmann, Schwyzer, and associates on synthesis, knowledge of the chemical structure of the intermedins has greatly progressed during the past three years.

After the determination of the complete structure of an acidic intermedin from hog [Harris & Roos (73, 74); Geschwind & Li (76); Geschwind *et al.* (77)], Harris & Lerner (71) and Harris (72) studied a basic peptide present in hog post-pituitary and endowed with the same biological activity. This peptide, discovered by Lee & Lerner (83) and called by them α-melanocyte stimulating hormone, represents 75 per cent of the melanocyte-stimulating activity of the gland. It has an acetylated α-amino group at one end and an α-carboxyl amide at the other end. The fragments obtained by enzymatic hydrolysis of the peptide were purified by paper electrophoresis and characterized according to the classical techniques of Sanger and of Edman and by degradation with carboxypeptidase. From these results, the complete structure of a hormone that contains 13 amino acid residues per molecule was obtained (Figure 1). This constitutes the first instance of a hormone that has an acetylated N-terminal group; this type of end-group has, however, already been reported in tobacco mosaic virus and cucumber virus-4 [Narita (84 to 86)]. On the other hand, the presence of an α-carboxyl amide end-group has already been demonstrated in oxytocin and vasopressin.

Intermedins of the β type have been sought in beef and in man. Ge-

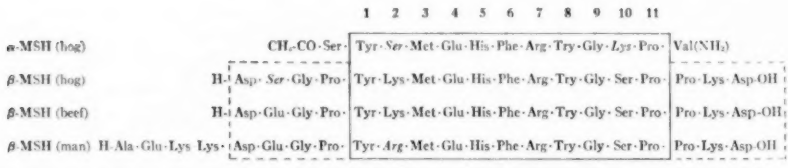
1 2 3 4 5 6 7 8 9 10 11
α-MSH (hog) CH3-CO-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val(NH2)
β-MSH (hog) H-Asp-Ser-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Try-Gly-Ser-Pro-Pro-Lys-Asp-OH
β-MSH (beef) H-Asp-Glu-Gly-Pro-Tyr-Lys-Met-Glu-His-Phe-Arg-Try-Gly-Ser-Pro-Pro-Lys-Asp-OH
β-MSH (man) H-Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Try-Gly-Ser-Pro-Pro-Lys-Asp-OH

FIG. 1. The melanocyte-stimulating hormones (α-MSH and β-MSH).

[5] Intermedin activity is expressed in terms of International Units (I.U.). Some workers use another measure defined by Shizume, K., Lerner, A. B., and Fitzpatrick, T. B. [*Endocrinology*, **54**, 553 (1954)]. One I.U. is equal to approximately 10^4 Shizume units [Cf. Geschwind, I. I. and Li, C. H., *J. Am. Chem. Soc.* **19**, 615 (1957)].

schwind, Li & Barnafi (78) have isolated the β-hormone from beef and have shown that it contains 18 amino acid residues like hog β-hormone; however, a serine residue replaces a glutamic acid residue (Figure 1). As a result, the physical properties are different, especially the isoelectric point, which is 7.0 instead of 5.8. The replacement of a charged residue by an uncharged one is unusual in the variations from one species to another. Harris (75) has studied the β-hormone of man; this is a slightly basic peptide with 22 amino acid residues per molecule. Starting with 5 mg. (about 2 μM), Harris has succeeded in determining the complete amino acid sequence by the usual techniques.

Figure 1 gives the results of the ensemble of investigations on the structure of intermedins. The relationship of the four substances is manifested by the presence of a sequence of 11 amino acid residues which vary only slightly from one to another: in the α-intermedin of hog, there is an exchange of residues 2 and 10, and in the β-intermedin of man there is a substitution of a lysine residue by arginine, a species variation already observed in vasopressins [Popenoe *et al.* (18)] and in cytochrome-*c* [Tuppy (87)]. This basic sequence is also found in the hog and sheep corticotropins that have a weak melanocyte-stimulating activity [Bell (88); Li (89)]. The presence of an α-amino group in the immediate proximity of the sequence seems to diminish the melanocyte-stimulating activity; the masking of this group, either by an acetyl radical or by peptides of different lengths, increases the activity. This is confirmed by the fact that the synthesized non-acetylated α-hormone is less active than the acetylated α-hormone.

The β-intermedins have a similar sequence of 18 residues, a serine residue replacing glutamic acid in beef β-hormone. This substitution, though outside the "active center," produces an unexpected decrease in activity of about 90 per cent [Li (70)]. The β-hormone of man possesses a supplementary N-terminal link, Ala·Glu·Lys·Lys. Is this the result of peculiarity of synthesis or is it attributable to the possibility that, in the release from a larger precursor, the bond Lys·Asp resists the action of a specific enzyme? Sela & Katchalski (90) have reported that the copolymers of lysine and glutamic acid are very resistant to the action of trypsin and that a sequence of Glu·Lys·Lys·Asp·Glu· could present a particularly strong resistance to certain enzymes. However, the precursor hypothesis is entirely arbitrary.

Certain conclusions in regard to activity-structure relationships have been confirmed by the use of synthetic products. Boissonnas and collaborators (91 to 94) and Hofmann *et al.* (95 to 98) have synthesized the α-melanocyte-stimulating hormone of pig, while Schwyzer & Li (99) and Schwyzer *et al.* (100) have prepared a protected peptide that has the amino acid sequence of beef β-hormone. The Sandoz workers have condensed N-acetyl-L-seryl-L-tyrosyl-L-seryl-L-methionyl-γ-benzyl-L-glutamate [Guttmann & Boissonnas (91)] with L-histidyl-L-phenylalanyl-L-arginyl-L-tryptophanyl-glycyl-ε-N-CBO-L-lysyl-L-prolyl-L-valyl amide [Boissonnas *et al.* (92)] and then obtained the hormone on elimination of the carbobenzoxy and

benzyl groups by hydrobromic acid in trifluoroacetic acid. The peptide was purified by countercurrent distribution and paper electrophoresis at pH 5.8. A product was obtained that was chromatographically and electrophoretically pure, titrated at 3300 I.U./mg., and possessed the physical and chemical properties of natural α-intermedin [Guttmann & Boissonnas (93)]. Guttmann *et al.* (94) have also shown that, when the α-amino group is not masked through an acetyl group, the melanocyte-stimulating activity decreases while the corticotropic activity increases.

In their work, Hofmann *et al.* (95 to 98) have condensed a pentapeptide carrying a carbobenzoxy group in the N-terminal position with an octapeptide that had the α-carboxyl amide group, and have obtained a tridecapeptide with the amino acid sequence of the α-hormone. Schwyzer and collaborators (99, 100) have synthesized an octadecapeptide corresponding to beef β-intermedin with the amino and carboxyl groups blocked by protecting radicals. The product possessed about 1 per cent of the activity of the natural substance (1.4 I.U./mg.). Table II presents the respective activities of the different peptides and hormones.

TABLE II

BIOLOGICAL ACTIVITIES OF MELANOCYTE-STIMULATING HORMONES

Peptide	Peptide Structure	Activity (I.U./mg.)
Pentapeptide	His·Phe·Arg·Try·Gly·	0.003
Hexapeptide	Glu·His·Phe·Arg·Try·Gly·	0.020
Octapeptide	Ser·Met·Glu·His·Phe·Arg·Try·Gly·	0.070
Bovine β-MSH*	Octadecapeptide	200.
Porcine β-MSH	Octadecapeptide	2000.
Porcine α-MSH	Tridecapeptide	3300.

* MSH = melanocyte-stimulating hormone.

Biological effects: metabolism.—Detailed information on the biological and clinical effects of intermedins can be found in the reviews by Lerner & Takahashi (101), Li (70), and Deutsch & Mescon (102).

The intermedins chemically closely resemble the corticotropins. They do not, however, possess corticotropic activity, whereas the corticotropins do have a small melanocyte-stimulating activity. When the acetyl group of the α-hormone is absent, corticotropic activity appears [Guttmann *et al.* (94)]. The physiological role of intermedins that have a marked pigmenting effect on batrachians has been sought in this class of vertebrates, but no clear conclusion can be drawn. Čehovič (103) has demonstrated with radioiodide an influence of the intermedins on the iodide metabolism in the frog, and Munday (104) has reported its action on carbohydrate metabolism in the toad; in the latter D-lysergic acid diethylamide can inhibit the secretion of intermedin [Burgers *et al.* (105)].

In mammals, even less data are available. Eviatar & Sulman (106) have found that the pituitaries of pigmented and albino rabbits contain the same quantity of hormone. The mechanism of secretion, the role, and the metabolism of the intermedins in higher vertebrates are unknown.

Corticotropin

Structure-activity relationships.—After determination of the structure of the corticotropins of pig [Shepherd *et al.* (107); White & Landmann (108)] and of sheep [Li *et al.* (109) Leonis *et al.* (110)], the study of beef corticotropin was undertaken by several workers [White & Peters (111); Li & Dixon (112); Li *et al.* (113)]. Li & Dixon (112) have purified the hormone by chromatography and countercurrent distribution. The corticotropin was obtained as a trichloroacetate, but the acid could easily be eliminated from the preparations by alkaline dialysis or adsorption on Amberlite IR-4B [Li (114)].

Beef corticotropin acts exactly like sheep corticotropin in chromatography on ion exchangers and in countercurrent distribution. The amino acid compositions are identical, and the same titration curves are obtained in 0.1 *M* KCl at 24.5° C. [Leonis & Li (115)]. The results obtained from degradation with phenylisothiocyanate and with carboxypeptidase, as well as from characterization of the peptides resulting from tryptic and chymotryptic hydrolysis, have permitted establishment of the complete formula of the hormone [Li, Dixon & Chung (113)]. The corticotropins of beef, pig, and sheep are each constituted of a single peptide chain of 39 amino acid residues. However, differences in the sequence are found from residue 25 to residue 32. The species variations are indicated in Figure 2. It is interesting to note that the "variable" sequence is rich in acidic residues (four residues out of eight) and is situated in the C-terminal part of the peptide chain, which can be removed without loss of biological activity [Shepherd *et al.* 107)]. The N-terminal sequence of 24 residues, which is indispensable for the preservation of activity, is identical in the hormones of the three species. The degradation of this part by aminopeptidase, trypsin, chymotrypsin, or fibrinolysin causes inactivation [Shepherd *et al.* (107); White (116); White & Gross (117)]. Boissonnas *et al.* (118) have synthesized a peptide of 20 residues that represents the N-terminal sequence, but it possesses only a very weak activity (2 to 3 I.U./mg.). Schwartz (119), on the other hand, has emphasized the internal correspondence between the sequences 12–23 and 24–35; each basic residue in the first sequence corresponds to an acidic residue in the second.

The chemical similarity between the intermedins and the corticotropins is striking. The "melanocyte-stimulating sequence" of 11 residues is found in the two families. However, the activity of corticotropins on melanocytes is weak. Treatment of corticotropin with dilute NaOH at 100° C. caused, on one hand, destruction of the corticotropic activity and, on the other, a 30- to 100-fold increase in the melanocyte-stimulating activity [Shepherd *et al.* (107)]. It is to be regretted that the degradation products were not isolated and characterized.

	25	26	27	28	29	30	31	32
Porcine corticotropin (A)	—Gly·	Ala·	Glu·	Asp·	Asp·	Glu·	Leu·	Ala—
Porcine corticotropin (β)	—Asp·	Gly·	Ala·	Glu·	Asp·	Glu·	Leu·	Ala—
Ovine corticotropin	—Ala·	Gly·	Glu·	Asp·	Asp·	Glu·	Ala·	Ser—
Bovine corticotropin	—Asp·	Gly·	Glu·	Ala·	Glu·	Asp·	Ser·	Ala—

FIG. 2. Species variations in the corticotropins.

Biological effects: metabolism.—Sayers *et al.* (120) have recently analyzed critically the existing data on the secretion of corticotropin by the pituitary under the influence of the hypothalamus and the action of the hormone on the adrenal cortex.

Guillemin *et al.* (121) have compared the different biological assays of corticotropin. Sayers' classical method, which is based on the decrease of adrenal ascorbic acid, and the more direct method of increase of corticosteroids in the plasma give the same values. However, the *in vitro* method of Saffran & Schally (121a), which is based on the production of corticosteroids by isolated adrenal glands, gives values distinctly higher. Several authors have recommended a simplification of the *in vivo* assay of Sayers: hypophysectomy is replaced by pharmacodynamic blocking of the pituitary with hydrocortisone or with 9-α-fluorohydrocortisone [Baillet *et al.* (122); Casentini *et al.* (123)]. A detailed study of the assay of corticotropin after subcutaneous injection has been published [Rerup (124)].

Recently, the secretion of corticotropin by the anterior pituitary has received attention; the secretion is stimulated by a hypothalamic hormone [Sayers *et al.* (120)]. According to McCann & Fruit (125), this hormone is vasopressin; actually, pure vasopressin does possess this biological activity [McCann & Fruit (125); McDonald *et al.* (126); Chauvet & Acher (127)]. However, the isolation of a substance that possesses a greater stimulating activity and yet no pressor action has led several workers to suggest the existence of a new hormone in the post-pituitary, the "corticotropin-releasing factor" [Schally & Guillemin (128); Schally *et al.* (129); Guillemin *et al.* (130, 131); Privat de Garilhe *et al.* (132); Rumsfeld & Porter (133)]. Schally *et al.* (129), starting with a commercial extract of post-pituitaries (Protopituitrin), have purified the active principle by paper chromatography. The isolated substance was still impure and gave upon hydrolysis 13 amino acids in variable proportions. Rumsfeld & Porter (133) have isolated from beef hypothalamus, by chromatography on Dowex-50 and countercurrent distribution, two substances which appear pure upon paper chromatography. The corticotropin-releasing factor, in contrast to vasopressin, was resistant to the action of 2.2 N HCl at 110° C. for 90 min. but was destroyed by pepsin [Schally *et al.* (129); Royce & Sayers (134)]. The preparation of larger quantities of the hormone by chromatography on carboxymethyl-cellulose [Schally & Guillemin (128)] will no doubt permit the isolation of a homogeneous substance and the comparison of its constitution with that of vasopressin.

The existence of a precursor of corticotropin, precorticotropin, has been suggested by Dasgupta & Young (135). Sterile alkaline extracts of beef anterior pituitaries that are inactive before treatment acquire a corticotropic activity when the medium is acidified (pH 3) or when urea (3 to 8 *M*) is added. The activation probably does not imply the rupture of covalent bonds. It is particularly attractive to consider the discovery of prohormones, similar to proenzymes, but a rigorous characterization of the precursors is necessary before acceptance of this point of view.

The mechanism of action of corticotropin on the adrenal glands is little known. According to Haynes (136), corticotropin indirectly activates an adrenal phosphorylase; the hormone, incubated with slices of beef adrenal glands, provokes an accumulation of adenosine-3′, 5′-monophosphate which in turn stimulates the activation of the phosphorylase. This is a specific action because the hormone does not activate liver phosphorylase. In a study of the action of corticotropin in the rat *in vivo*, Hokin *et al.* (137) did not observe an increase in the incorporation of radiophosphorus in the adrenals. The hormone also seems to favor the formation of a precursor of corticosteroids [Koritz *et al.* (138)]. It is interesting to note along this line that the decrease in adrenal ascorbic acid, which in the mammals accompanies the decrease in cholesterol after an injection of corticotropin, does not take place in the chicken [Howard & Constable (139)]. Finally, Turchetto & Cenciotti (140) have observed that the hormone increases the glutamic acid-oxaloacetic acid transaminase in rat serum. It is not yet possible to define with any precision the chemical mechanism of action of corticotropin.

Thyrotropin

Isolation.—A complete review of the chemistry and physiology of thyrotropin or thyroid-stimulating hormone has been recently published by Sonenberg (141). Although the hormone has not yet been isolated in the pure state, preparations about 2000 times more active than anterior pituitary acetone powders have been obtained, especially through the use of cellulose ion exchangers. The thyrotropins of beef, sheep, whale, and man have been purified, but the greatest progress has been realized in the case of the beef hormone as a result of the work of Condliffe, Pierce, and their collaborators.

Condliffe & Bates (142) and Condliffe *et al.* (143) have pursued their investigations on the use of diethylaminoethyl-cellulose and carboxymethyl-cellulose in the purification of thyrotropin. A saline extract of anterior pituitary acetone powder (0.03 u./mg.) was fractionated with acetone; the product obtained (1 u./mg.) was chromatographed on a column of diethylaminoethyl-cellulose with an ionic strength gradient (0.005 to 0.2) made with glycine buffers at pH 9.5. The active fraction (5 u./mg., yield 50 per cent) was rechromatographed under the same conditions; then the purified product (10 to 15 u./mg.) underwent chromatography on carboxymethyl-cellulose (equilibrated with a 0.01 *M* phosphate buffer, pH 6) and an elution gradient with *M* NaCl. Preparations of 20 to 50 u./mg. were thus obtained. The hor-

mone was less stable in the highly purified state than in the presence of contaminating proteins. The results of the chromatography also suggest the presence of two active constituents in the extracts.

Carsten & Pierce (144) and Pierce *et al.* (145) utilized acetone fractionation and distribution in a system of n-butanol-0.05 *M* *p*-toluene sulfonic acid to obtain a crude product of 5 u./mg. The active material then underwent two successive chromatographic steps on diethylaminoethyl-cellulose under the conditions described above. Starting with 1 kg. of beef anterior pituitaries, these authors isolated 5 to 6 mg. of a powder titrating at 40 u./mg. Upon zone electrophoresis of this product on starch, two active substances were separated; it is not yet possible to decide whether one of the substances was derived from the other by degradation, whether the active principle is associated with two slightly different proteins, or whether two hormones exist in the gland. The agreement between the results obtained by the two groups of workers utilizing different chemical procedures and different biological assays is very satisfactory.

The purification of sheep and whale thyrotropins has been undertaken by Pierce *et al.* (146) with the same techniques used to obtain the beef hormone. In the course of electrodialysis, the three hormones were retained by the cellophane membrane. By fractional precipitation with ammonium sulphate and metaphosphoric acid, followed by chromatography on Amberlite IRC-50, Ellis (147) has obtained from sheep a thyrotropin of 13 u./mg. Heideman *et al.* (148) find that human thyrotropin behaves like the beef hormone in chromatography on Amberlite IRC-50 and on carboxymethyl-cellulose.

Chemical properties.—The chemical properties of thyrotropin are not well-known because the substance has not yet been obtained in the pure state. Pierce & Carsten (149), using a highly purified preparation (30 u./mg.), have estimated the molecular weight to be about 26,000 to 30,000. The authors have studied, on electrodialysis, the passage of different proteins of known molecular weight across a stretched and a non-stretched cellophane membrane; with this calibration, it was possible to attribute to thyrotropin an approximate molecular weight. The sedimentation constant is of the order of 2.5 to 3.0 S [Condliffe *et al.* (143)]. Recent determinations of the molecular weight and the sedimentation constant indicate values about three times higher than those previously obtained with less pure preparations. Only the definitive purification of the hormone will permit the resolution of this disagreement by making it possible to measure the molecular weight by a chemical method.

Condliffe *et al.* (143) have treated purified thyrotropin (20 u./mg.) with dinitrofluorobenzene and have obtained, after hydrolysis, dinitrophenyl-phenylalanine and dinitrophenyl-threonine, which suggest the presence of free α-amino groups in the molecule; however, upon starch electrophoresis of these preparations, two constituents could be seen, and the experiment should be repeated with a homogeneous substance. Acetylation with acetic

anhydride, which blocks various groups, especially the amino groups, destroys the biological activity [Sonenberg & Money (150)].

Biological effects: metabolism.—Among the numerous radiobiological assays which have been proposed for the thyroid-stimulating hormone, three *in vivo* methods have been used to follow the purification of the hormone. Bates & Cornfield (151) estimate the decrease in I^{131} in the chick thyroid and several workers have used this procedure with satisfactory results [Condliffe *et al.* (143); Frey & Albert (152)]. Others have assayed the uptake of P^{32} by the chick thyroid [Pierce *et al.* (145); Ellis (147)]. Finally, the uptake of I^{131} by mouse thyroid has served as the basis of a more physiological assay, since the receptor animal is of the same class of vertebrates as those from which the hormone is prepared [Querido *et al.* (153); Fontaine (154)]. Several *in vitro* methods that use thyroid slices and measure either the increase in weight [Bakke *et al.* (155)] or the incorporation of P^{32} [Florsheim *et al.* (156)] seem promising, and, as they permit a rapid estimation of the biological activity, they could considerably accelerate the purification of thyrotropin.

The existence of a hypothalamic hormone, which stimulates the secretion of thyrotropin by the anterior pituitary (thyrotropin-releasing factor), has been reported [Yamamoto (157); Nishi (158)]. D'Angelo (159) and Bogdanove (160) have modified the secretion of the hormone by creating lesions in the rat hypothalamus; the experimental results indicate that the hypothalamus controls the activity of the "pituitary-thyroid system" by influencing the production and the secretion of thyroid-stimulating hormone by the pituitary. Furthermore, the presence of thyrotropin in the blood has been demonstrated, and variations in its secretion, as a function of different factors, has been studied [Fontaine (161); McKenzie (162)].

Somatotropin

Isolation.—Somatotropin, or growth hormone, has been purified from the anterior pituitaries of various mammalian species. Papkoff & Li (163) have pursued their work in this field in the isolation of the somatotropin of whale (humpback whale). The frozen anterior pituitaries were homogenized in a solution of calcium hydroxide at pH 10. The hormone was precipitated from the extract by half-saturated ammonium sulfate at pH 7.0, and the fraction that precipitated between 0.2 and 0.4 saturation was retained. This material then underwent chromatography on Amberlite IRC-50, and the active product was purified further by fractional precipitation with ethanol. About 0.1 gm. of the hormone was obtained from 100 gm. of glands. The hormone appeared to be homogeneous on electrophoresis and ultracentrifugation. The molecular weight, calculated from sedimentation ($S_{20,w} = 2.84S$) and diffusion ($D_{20,w} = 6.56 \times 10^{-7}$), is 39,000; the isoelectric point is pH 6.2. Papkoff & Li (164) have also purified sheep somatotropin by the same techniques. The product was homogeneous on chromatography, electrophoresis, and ultra-

centrifugation. The molecular weight, calculated from the sedimentation ($S_{20,w} = 2.76S$) and diffusion ($D_{20\,w} = 5.25 \times 10^{-7}$), is 47,000, and the isoelectric point is pH 6.8. Ottaway (165) has obtained a purified preparation from pig glands by aqueous extraction at pH 7.8 and subsequent fractional precipitation with ammonium sulfate and ethanol.

Raben (166, 167) has purified human somatotropin. Anterior pituitary acetone powder was treated with a mixture of hot acetic acid-acetone; the hormone was then precipitated with ether. The precipitate was redissolved in dilute acetic acid, and the contaminating corticotropin and intermedin were eliminated by adsorption on oxycellulose. The pH was adjusted to 8.5, and, after elimination of the resultant precipitate, the hormone was precipitated by alcohol (yield in weight was 4 per cent). The somatotropin thus isolated was homogeneous on chromatography on carboxymethylcellulose with a gradient of $CaCl_2$ in 2 *M* acetic acid. Because a part of the material remained irreversibly adsorbed onto the column, the author has reservations in regard to the purity of the product. Lewis & Brink (168) have succeeded in crystallizing the human hormone, first by fractionating extracts of the entire pituitaries with ethanol, followed by addition of alcohol, to 25 per cent final concentration, to a solution of the product in 0.1 *M* KCl, pH 7.5. The hormone could also be obtained from the crude extract by chromatography on diethylaminoethyl-cellulose with a yield of 10 per cent. Alterations have been observed at pH 4 and at pH 10, but without loss of biological activity. Lazo-Wasem & Graham (169) and Lazo-Wasem *et al.* (170) have shown that the stability of somatotropin as a function of pH depends on the initial method of extraction, alkaline extraction rendering the hormone unstable at low pH (pH 3).

The results obtained to the present time indicate that, while the isoelectric points of the several somatotropins differ but little (from pH 5.5 to 6.8), the molecular weights differ greatly, from 25,000 to 27,000 for man, monkey, and pig, to 39,000 for whale and 47,000 for beef and sheep.

Chemical properties.—The study of the chemical structure of the different somatotropins has revealed the existence of two types of molecules: one has a single N-terminal amino acid, phenylalanine, and is found in man, monkey and whale [Parcells & Li (171); Heijkenskjöld (172)], and the other has two N-terminal amino acids, phenylalanine and alanine, and is found in beef and sheep [Papkoff & Li (164)]. The N-terminal sequence for the human hormone is Phe·Ser·Thr., and the N-terminal sequences for the beef hormone are Phe·Thr·Ala and Ala·Phe·Ala [Parcells & Li (171)]. Through the use of carboxypeptidase and hydrazinolysis, it has been demonstrated that all of the somatotropins have one single C-terminal residue per molecule, phenylalanine. The C-terminal sequence of this hormone is Ala·Leu·Phe in sheep, Leu·Ala·Phe in whale, (Ala, Gly)Phe in monkey, and Leu·Phe in man [Li *et al.* (173)]. More experimentation is necessary to determine the special bonds between the two peptide chains in the beef and sheep somatotropins.

Geschwind & Li (174) have attached guanidine groups to the ε-amino

groups of beef somatotropin without loss of activity; the blocking of the α-amino groups of the guanidine-substituted hormone also does not modify the activity.

Biological effects: metabolism.—Knobil & Greep (175) and Russell & Wilhelmi (176) have analyzed in detail the physiological properties of growth hormone. Differences in the biological action of somatotropins of different species have been observed by use of hypophysectomized rats [Li *et al.* (177)]. Daily injections of the beef or whale hormone provoked a regular increase of weight during 90 days, whereas an identical treatment with the human hormone stimulated a weight increase only the first 10 days. Hayashida & Li (178) have obtained in the rabbit a specific antiserum against the beef hormone; in contrast, the human hormone does not have any antigenic properties in the rabbit [Heijkenskjöld & Gemzell (179)].

The mode of action of somatotropin is unknown. Certain authors claim that the hormone favors the biosynthesis of nucleic acids [Cater *et al.* (180)] and others that it stimulates directly the formation of microsomal proteins [Balis *et al.* (181)]. On the other hand, the production as well as the secretion of somatotropin could be, themselves, under the influence of a hypothalamic factor, analogous to the corticotropin-releasing factor and thyrotropin-releasing factor, which act respectively upon the secretion of corticotropin and thyrotropin [Del Vecchio *et al.* (182)].

Prolactin

Purification and structure.—A general review on prolactin has been recently published by Li (70). After the isolation of sheep prolactin by isoelectric precipitation and by countercurrent distribution, Cole & Li (183) tested the homogeneity of this substance by passing it through a countercurrent distribution of, first, 900 transfers in a butyl cellosolve-sucrose solution system at pH 7 and, then, 1500 transfers in a 2-butanol-0.35 per cent dichloroacetic acid system. One single constituent was found. When, however, the prolactin had been lyophilized at pH 8 prior to the distribution, several active fractions were found in the second solvent system. The molecular weight of prolactin, based on sedimentation and diffusion, is 24,200, a value in agreement with that obtained by chemical means [Li *et al.* (184)].

The oxidation of disulfide bridges by performic acid does not break the molecule [Li (185)]; similarly, the reduction of these bonds by mercaptoethanol at pH 8.9 in 10 *M* urea and then alkylation of the thiol groups by iodoacetamide led to a single fragment [Li & Cummins (186)]. The molecule appears to be constituted by a single peptide chain: the N-terminal sequence is Thr·Pro·Val·Thr·Pro., and the C-terminal sequence, as determined by the action of carboxypeptidase on the reduced hormone and by hydrazinolysis of the oxidized hormone, is Tyr·Leu·Asp(NH_2)-CyS– [Li & Cummins (186)]. The reduction of the three disulfide bridges by mercaptoethanol abolishes the biological activity. Acetylation of eight amino groups also inactivates the hormone, but guanidination of the 10 ε-amino groups does not

affect the lactogenic activity. When all of the ϵ-amino groups are guanidinated and the α-amino group acetylated, the activity remains unchanged, indicating that the latter group is not necessary for the biological activity. Oxidation of the N-terminal threonine by periodate also does not result in loss of biological activity, thus confirming the preceding conclusion [Geschwind & Li (174); Li & Cummins (186)].

Biological effects.—Grosvenor & Turner (187) have proposed a biological assay based on the degree of proliferation of pigeon crop as a function of prolactin dose; the standard and the unknown can be assayed on the same animal by use of the two crop-sacs.

The role of the post-pituitary in the secretion of prolactin by the anterior pituitary, especially the action of oxytocin, has been studied [Benson & Folley (188); Stutinsky (189); McCann *et al.* (190)]. Certain hypothalamic lesions in the suckling rat bring about a block of both milk secretion and milk ejection; the injection of exogenous oxytocin retards these effects [McCann *et al.* (190)]. The chemical mechanism of the action of prolactin on mammary tissue is unknown; according to Read & Moore (191), however, prolactin seems to inhibit the degradation of coenzyme A.

Pituitary Gonadotropins

Interstitial cell-stimulating hormone.—The purification of interstitial cell-stimulating hormone or luteinizing hormone has been undertaken by several workers, especially from sheep glands but also from the glands of other mammalian species.

Ellis (147) treated sheep pituitaries with 0.1 *M* ammonium sulfate at pH 7.5 and then precipitated the interstitial cell-stimulating hormone simultaneously with somatotropin and thyrotropin by raising the ammonium sulfate concentration to 2.2 *M*. The latter two hormones were eliminated by ammonium sulfate and metaphosphoric acid; the desired hormone was then purified by adsorption on Amberlite IRC-50 at pH 8.0 and elution with sodium chloride. The product obtained appeared to be homogeneous in moving boundary electrophoresis. The Sanger technique indicated mainly the presence of threonine and serine in the N-terminal position with a molar ratio of 1:1. Steelman & Segaloff (192) have also obtained a purified hormone from sheep glands, first by fractionation of extracts with alcohol and ammonium sulfate and then by utilization of chromatography on carboxymethyl-cellulose. Squire & Li (193, 194) treated pituitary acetone powder with 0.5 per cent NaCl. The material obtained was fractionated with sodium salicylate and ammonium sulfate, and the active product, after chromatography on Amberlite IRC-50, was then submitted to zone electrophoresis on starch. Two active components (α and β), representing about 12 per cent of the initial activity, were obtained on the Amberlite IRC-50 chromatography. The β component appeared homogeneous in chromatography and on ultracentrifugation but was heterogeneous on electrophoresis. The isoelectric point was pH 7.3, and the molecular weight, based on sedimentation and diffusion, was 30,000.

Jutisz & Squire (195) have found five active components in sheep hormone by cellulose column electrophoresis of a purified preparation. Leonora *et al.* (196) have obtained a preparation of sheep interstitial cell-stimulating hormone which was only very slightly contaminated with follicle-stimulating hormone; the former gonadotropin was first adsorbed on an anion-exchange resin (Amberlite IR-4B) and then a cation-exchange resin (Amberlite IRC-50).

The purification of the human hormone has also been undertaken [Steelman *et al.* (197); Segaloff & Steelman (198); Rigas *et al.* (199)]. Steelman and collaborators (197) have extracted the pituitary acetone powder with an acetate buffer of pH 5 in 40 per cent ethanol. The active substance was precipitated from this extract by increasing the ethanol concentration to 80 per cent. The product contained a large amount of follicle-stimulating hormone, but the two gonadotropins could be separated by chromatography on diethylaminoethyl-cellulose by use of volatile buffers. Rigas *et al.* (199) have obtained a gonadotropic preparation by fractional precipitation with alcohol and ammonium sulfate, but they did not separate the two hormones.

The assay of the different gonadotropins has been criticized by Apostolakis & Voigt (200). Lostroh *et al.* (201) have shown that the classical assay of interstitial cell-stimulating hormone, based on the increase in weight of rat ventral prostate, is valid only for certain strains of animals, since prolactin and somatotropin also have this effect in the Sprague-Dawley rat. The injection of the hormone into the immature female rat, first treated with pregnant mare serum gonadotropin, provokes a superovulation; Zarrow *et al.* (202) report that the increase in the number of liberated ova could serve as the basis of an assay 150 times more sensitive than that based on increase of prostate weight. The feather of the weaver finch [Segal (203)] or the uptake of P^{32} by the chick testicle [Florsheim (204)] have also been proposed as tests, but the latter assay is not specific for interstitial cell-stimulating hormone since all of the gonadotropins stimulate this uptake. As in the case of several other anterior pituitary hormones, the secretion of this hormone appears to be under the control of the hypothalamus, since lesions in this region block the formation of corpora lutea [Van Dyke *et al.* (205)].

Follicle-stimulating hormone.—Follicle-stimulating hormone has been purified from the pituitaries of sheep, pig, and man. After extraction of sheep glands, Ellis (147) collected the fraction that precipitated at pH 7.7 between 2.2 and 3 *M* ammonium sulfate; from this fraction, the proteins precipitable by 3 *M* ammonium sulfate at pH 4 and metaphosphoric acid at pH 3 were eliminated and the follicle-stimulating hormone then precipitated in turn with 35 per cent alcohol at pH 4. The hormone was then chromatographed on diethylaminoethyl-cellulose and submitted to zone electrophoresis on starch. The product obtained was 600 times more active than the initial crude extract, and appeared homogeneous in moving boundary electrophoresis. The isoelectric point of the sheep hormone, as determined by electrophoresis on starch, is pH 4.6 [Raacke *et al.* (206)]. Steelman & Segaloff (192) and Steelman (207) have purified the pig hormone by ammonium

sulfate fractional precipitation and repeated chromatography on diethylaminoethyl-cellulose and hydroxyapatite. The highly purified product loses its activity upon lyophilization.

The human hormone has also been purified [Li (208); Segaloff & Steelman (198)]. Li (208) extracted the pituitary powder with a calcium hydroxide solution at pH 10.3, eliminated somatotropin by precipitation with 1.9 *M* ammonium sulfate, and then precipitated the follicle-stimulating hormone by increasing the ammonium sulfate concentration to 3 *M*. After adsorption of contaminating proteins on Amberlite IRC-50 at pH 5.1, the hormone was again purified by ammonium sulfate fractional precipitation. Starting with 10 gm. of pituitary powder, 100 mg. of a product 15 times more active were obtained. The hormone was still contaminated with interstitial cell-stimulating hormone, but the preparation contained no thyrotropin, corticotropin, prolactin, or somatotropin. Segaloff & Steelman (198) have obtained a purified product by fractional precipitation with ethanol and adsorption on diethylaminoethyl-cellulose. The hormone has been found in plasma [Antoniades *et al.* (209)] and in urine [Segaloff & Steelman (198)] of postmenopause women.

Hollander, Hollander & Tull (210) have shown, by use of labeled testosterone, that follicle-stimulating hormone, acting on slices of dog ovaries, stimulates the transformation of testosterone into estradiol. This stimulation is also observed after the addition of chorionic gonadotropin. Fukuda (211) has reported that secretion of the hormone seems to be under hypothalamic control, as electrical stimulation of the hypothalamus increases the secretion, and lesions in this organ decrease secretion.

PLACENTAL HORMONES

Human Chorionic Gonadotropin

Human chorionic gonadotropin has been extracted from the urine or from the chorionic membrane of pregnant women. The hormone was extracted from urine by Butt (212) by adsorption with benzoic and tungstic acids. After elimination of the acids with absolute alcohol and acetone, the hormone was precipitated with 75 per cent alcohol at pH 7. Kikutani (213) has extracted the hormone from the placental chorionic membrane. After studying the action of enzymes and of ketene on the hormone, this author concluded that the carbohydrate moiety is not essential for the biological activity but that tyrosine plays a major role in this activity.

The sensitivity of the hormone to the action of various physical and chemical factors has been studied. At room temperature, it loses its activity at either low or high pH, but is stable in neutral solution; at low temperatures the resistance of the hormone to extremes of pH is increased [Banik (214); Bourrillon *et al.* (215)]. Silver nitrate, mercuric chloride, sodium bisulfite, *m*-dinitrobenzene, formaldehyde, and performic acid abolish its biological activity; fluorodinitrobenzene inactivates it partially, but iodine in acid solution is without effect [Bourrillon *et al.* (215); Banik (216); Banik & Chakravarti (217)].

The responses of rat ovary and uterus as a function of hormone dose vary differentially with human chorionic gonadotropin or the urinary gonadotropin of pituitary origin; a biological distinction between these hormones has been proposed by Albert & Kelly (218). The biological assay of human chorionic gonadotropin, based on increase in weight of the uterus of immature mice, has been evaluated by several authors [Claringbold & Lamond (219); Lamond (220); Rosemberg *et al.* (221); Cunningham & Brown (222)].

Pregnant Mare Serum Gonadotropin

The purification of pregnant mare serum gonadotropin has greatly progressed, thanks to the efforts of Legault-Démare, Clauser & Jutisz (223), Bourrillon & Got (224), and Bourrillon *et al.* (225). Legault-Démare *et al.* (223), after adjustment of the plasma pH to 5, collected the fraction that precipitated between 50 and 80 per cent alcohol; a crude product of 300 to 500 I.U./mg. and representing 70 to 90 per cent of the original activity was thus obtained. By adsorption of this on a Permutite column, and then elution with 0.2 *M* Na_2CO_3 in 15 per cent ethanol, a material of 1000 to 1500 I.U./mg. was obtained. The contaminating proteins in this eluate were eliminated by adsorption on barium carbonate. The final product thus isolated was of 10,000 to 13,000 I.U./mg., and the overall yield was 60 to 70 per cent.

Bourrillon & Got (224) and Bourrillon *et al.* (225) have started with preparations of 800 to 1000 I.U./mg. The material was extracted at low temperatures with 50 per cent alcohol in a 0.066 *M* acetate buffer of pH 4.8; after elimination of the insoluble inactive material, the pH of the extract was raised to 8.5 and the concentration of alcohol to 55 per cent; an inactive deposit was eliminated, and the hormone was precipitated by increasing the alcohol concentration to 80 per cent. This product (4000 to 6000 I.U./mg.), upon zone electrophoresis on starch at pH 8.6, gave two components, only one of which was active. This active protein was isolated and precipitated by acetone; it appeared to be pure upon moving boundary electrophoresis at pH 8.6, 4.5, 2.5, and 1.95, and its isoelectric point was 1.8. The specific activity was 8000 I.U./mg. The hormone is a glycoprotein, the protein part of which represents only 40 per cent of the molecule. The analysis of the carbohydrate moiety revealed 20.5 per cent monosaccharides (galactose, mannose, glucose, fucose, rhamnose), 14.1 per cent hexosamines (glucosamine, galactosamine), and 10.4 per cent sialic acids [Bourrillon *et al.* (225)]. In the serum of pregnant mares, the hormone is associated with inactive proteins, certain physical and chemical properties of which have been determined [Bourrillon *et al.* (226, 227)].

The *in vivo* biological assay of placental gonadotropins (human chorionic gonadotropin and pregnant mare serum gonadotropin) has been criticized by Hamburger (228). Suarez-Soto & Legault-Démare (229) have proposed an *in vitro* assay for pregnant mare serum gonadotropin: the hormone, when incubated with slices of rat ovary, stimulates the release of ketosteroids, the quantity produced being proportional to the logarithm of the hormone dose.

PANCREATIC HORMONES

Insulin

Isolation.—A new method for the preparation of insulin has been recently reported by Pettinga (230). Beef, pig, or dog pancreas is extracted with an acidic alcoholic solution, and the extracts are precipitated by alcohol and ether at pH 3; the precipitate is redissolved in dilute sulfuric acid, and the solution is seeded with fibrils prepared from crystalline insulin according to the method of Waugh. The hormone precipitates as fibrils and can subsequently be crystallized.

The behavior of beef insulin in partition chromatography with systems of 2-butanol-0.1 *N* acid has been studied by Carpenter (231): the higher the distribution constant of the acid in the 2-butanol-water system, the higher is the distribution constant of the hormone. Paper chromatography of insulin under appropriate conditions has been performed by several workers [Muić *et al.* (232); Bouman & Homan (233)]; these methods permit the separation of insulin and ribonuclease or of insulin and glucagon.

Structure-activity relationships.—The brilliant work of Sanger and his collaborators has led to determination of the complete structure of the insulins of beef, pig, horse, sheep, and whale. Ishihara *et al.* (234) have utilized the degradation procedures of the English workers in the study of the insulins of sperm- and sei-whales. The structure found for the sperm whale insulin corresponded to that determined by Sanger and collaborators. A difference was found, however, between the insulins of sperm- and sei-whales in the sequence of residues 8 to 10 in chain A: the sequence Thr·Ser·Ileu is found in the former and the sequence Ala·Ser·Thr· in the latter. It is interesting to note that position 10, which in other mammalian species is occupied by either valine or isoleucine (species variation also observed in the hypertensins), is replaced by threonine in the sei-whale.

The splitting of the insulin disulfide bridges by sodium sulfite has been studied by Cecil & Loening (235). At pH 2.8 to 5.5, only one of the bridges is split, but, at pH 6.5 to 7.0 in 8 *M* urea, the three bridges are broken. The reduction by glutathione is accelerated by certain enzymes of rat liver [Narahara & Williams (236)]. The hydrolysis of insulin by ion-exchange resins (Amberlite IR-120 and Amberlite IR-4B) is very different from the hydrolysis by mineral acids; the peptide bonds broken are in the C-terminal part of the B chain, outside of the ring formed by the disulfide bridges [Davies & Harris (237)]. The determination of the C-terminal residues of the molecule by hydrazinolysis has not been improved by the utilization of methylhydrazine [Bradbury (238)]. The synthesis of various peptides representing insulin fragments has been undertaken [Chillemi *et al.* (239); Holland & Cohen (240)].

The biological activity of insulin is not affected by guanidination of the unique lysine residue into homoarginine [Evans & Saroff (241)]. However, this activity disappears when the carboxyl groups are esterified [Nicol

(242)]. On the basis of the results obtained by degradation of the molecule with leucine aminopeptidase Smith *et al.* (243) have concluded that the first six residues of the B chain are not essential for the hormonal activity.

Several chemical assay methods for insulin have been proposed. Grodsky (244) has made use of the fibril-forming property of the hormone; when pancreatic extracts are seeded under appropriate conditions with freshly prepared fibrils, insulin precipitates almost quantitatively and can be estimated by ultraviolet absorption; the source of the insulin fibrils used for seeding is unimportant. This procedure has been applied as an assay in beef, pig, cat, and rat pancreas, and the values found were comparable to those obtained with biological methods. Fenton (245) has separated insulin by paper chromatography with the system 2-butanol- 1 per cent acetic acid; he used bromocresol green as the stain, and, after elution of the insulin spot, estimated the hormone by photometry; the results were in agreement with those obtained by the mouse-convulsion test.

Metabolism and mode of action.—The secretion of insulin by the pancreas may be stimulated by growth hormones [Engel *et al.* (246)] and by sugars which can be utilized by the organism [Pozza *et al.* (247)]. In blood, insulin could be associated with proteins [Antoniades (248)].

Mirsky & Perisutti (249) have demonstrated the existence in liver of an enzymatic system that is relatively specific for the destruction of insulin. The purification of this enzyme system has been undertaken. Strässle (250) has obtained an active fraction from rat liver by precipitation of the extract with alcohol at pH 7.2; I^{131}-iodinated insulin is used as substrate. Tomizawa & Halsey (251) have started with beef liver and have fractionated the extract by chromatography on Amberlite XE-64, precipitation with ammonium sulfate and alcohol, and zone electrophoresis; the enzyme obtained was homogeneous on ultracentrifugation and in paper electrophoresis. These insulin derivatives, which are not degraded *in vivo*, are in general those which do not exhibit biological activity [Karasek *et al.* (252)]. However, insulin is also hydrolyzed by extracts of organs other than liver, especially by extracts of the anterior pituitary and of the diaphragm [Narahara & Williams (253, 254)]; further work is necessary before the existence of a specific "insulinase" can be accepted.

Work on the mechanism of insulin action continues in several laboratories. Dunn *et al.* (255) claim that hypoglycemia induced by insulin is caused by an increase in glucose fixation by the tissues and not by a decrease in the release of hepatic glucose; actually, when the body glucose pool is labeled with C^{14}-glucose and insulin is infused, there is no change in the specific activity of the plasma glucose. Levin & Weinhouse (256) have observed that, under the influence of insulin, C^{14}-glucose is incorporated more rapidly into the glycogen of muscle. Insulin antibodies appear to inhibit the action of insulin on the diaphragm [Wright (257)]. The effect of the hormone on the transport of sugars across the cell membrane has been studied [Park *et al.* (258); Sacks & Smith (259)].

The action of insulin, *in vitro*, on the metabolism of adipose tissue in rat has been studied [Winegrad & Renold (260, 261); Ball *et al.* (262); Hagen *et al.* (263); Hausberger (264)]. Insulin stimulates the fixation and oxidation of glucose and its conversion into lipids; the incorporation of C-6 into the fatty acids is greater than that of C-1, but insulin stimulates lipogenesis from either C-1 or C-6 to the same extent [Winegrad & Renold (260, 261)]. The hormone, when added to slices of mammary gland from suckling rats, stimulates the formation of CO_2 from C-1 and fatty acids from carbons 1, 3, 4, and 6 of glucose [Abraham *et al.* (265); McLean (266)]. Insulin also favors the release of fatty acids from the tissues into the blood [Bierman *et al.* (267)].

Insulin stimulates amino acid transport into the isolated rat diaphragm [Kipnis & Noall (268)] and incorporation of these amino acids into proteins [Manchester & Young (269)]. Induction of tryptophan peroxidase of rat liver by insulin has been reported [Schor & Frieden (270)]. The hormone appears to act also on ion transport; rat diaphragm, soaked in physiological saline, has a lower sodium content and a higher potassium content when insulin is added to the solution [Creese *et al.* (271)].

Glucagon

The complete structure of glucagon is now available, thanks to the work of Behrens and collaborators. Behrens & Bromer (272) have published a very pertinent review on the chemical and biological properties of this hormone. The specific break of the peptide chain at the carboxyl group of the single tryptophan residue was performed with N-bromosuccinimide. [Patchornik *et al.* (273)]. The C-terminal sequence Try·Leu·Met·Asp·Thr was therefore split and released the expected tetrapeptide Leu·Met·Asp·Thr. The proteolysis of glucagon by enzymes in the rat liver has also been studied [Kenny (274)].

Several biological assays for glucagon have been proposed: either *in vivo*, based on its hyperglycemic action in fowl [Beekman (275)], or *in vitro*, based on its stimulation of glycogenolysis in liver slices [Tyberghein & Williams (276); Vuysteke & de Duve (277)]. The physiological relationships between glucagon and insulin have been studied by Miahle (278). Hyperglycemia induced by glucagon is not caused by inhibition of the utilization of glucose by the tissues [Tomizawa *et al.* (279)]; in the rat, the hormone stimulates an increase of oxygen consumption and of metabolism [Davidson *et al.* (280)]. The action of glucagon on the external secretion of the pancreas [Necheles *et al.* (281)], on gastric secretion [Robinson *et al.* (282)], on lipemia [Albrink *et al.* (283)], and on gestation [Curry & Beaton (284)] have also received attention.

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BIOCHEMISTRY OF CANCER[1,2]

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It is perhaps pointless to begin this particular chapter each year with a statement about the magnitude of the task of preparation. It is apparent that the field of cancer research is practically limitless in breadth and is, in fact, a major contributor to the extension of our present limits of concept and of experimentation. Suffice it to say that, in accordance with the request of our editors, the scope and emphasis of the present review have been determined largely by the interests of the reviewers and by what they believe to be also the current interests of a fair number of their readers.

Carcinogenesis

Present knowledge and concepts of carcinogenesis have been presented in two recent symposia (1, 2) and were the subject of the cancer chapter in last year's *Annual Review of Biochemistry* (3). Our current lack of understanding of the process indicates how little we know about the fundamental processes of the living cell, normal as well as neoplastic.

Genetic aspects.—Genetics and cancer have been considered in three conferences (4, 5, 6); the reader is referred to these for discussions of specific aspects of this subject.

Recent studies on chromosome aberrations in tumors (7, 8) have given new scope to the concept of cancer as a somatic mutation. However, although chromosomal rearrangements toward aneuploidy may be associated with, or even aid in, progression of a tumor toward autonomy (9, 10), such changes are not necessarily associated with early stages of tumor growth and do not appear to be a likely cause, or even a prerequisite, of transformation to neoplasia. Chromosomal instability and variability may be merely one expression of "the phenotype of this kind of cell" [Schultz (4)].

It seems clear, however, that any theory of carcinogenesis must ultimately account for the heritable nature of the process by some effect on the cell's

[1] This chapter represents a general coverage of the literature up to the latter months of 1959. For helpful discussions and for criticisms on parts of the manuscript, the authors are indebted to Drs. Bruce Ames, R. W. Brockman, Clyde Dawe, Leon Heppel, Royce Lockart, and Robert Love. Sincere appreciation is also expressed to Miss Anne McCullough for aid in the literature search and to Mrs. Jane Bell and Mrs. Elizabeth O'Malley for valuable assistance in the preparation of the manuscript.

[2] The following abbreviations are used in this chapter: AMP for adenosine monophosphate; GMP for guanosine monophosphate; IMP for inosine monophosphate; XMP for xanthosine monophosphate.

heredity mechanisms. Our concepts as to how such an effect could be mediated have both broadened and become more specific, as indicated, for example, by the proceedings of three recent symposia on heredity (11), development (12), and somatic cell variation (13). It is now possible to visualize "feedback" control, or "repression," of enzyme synthesis (12) as functioning at the genic level, since the activity of a series of linked genes can apparently be affected simultaneously, as in the control of the enzymes for biosynthesis of tryptophan (14) and histidine (15). In a recent discussion of the subject (14), inherently chromosomal genes, controlling protein structure, are distinguished from "regulating" genes, affecting the expression of "structural" genes and controlling protein synthesis; the relation to the concept of suppressor genes (16) is evident. Modification of the factors that control enzyme synthesis would affect cell metabolism; if the enzyme systems in question were those involved in DNA synthesis and cell division, capacity for cell proliferation might also be affected (17).

For modification of the cell's heredity by new elements that interact with the chromosome and replicate with cell proliferation, the best models can probably be found in work with bacterial systems; from this it has become apparent that various kinds of subcellular particles can function as possible replication units and carriers of heritable information (11, 18, 19). The current trend in thinking is illustrated by the concept of episomes, as discussed by Jacob and co-workers (6, 14, 20). Episomes are defined as genetic elements, of as yet unknown chemical constitution, that can exist in the cell either as autonomous replication units or as units attached to and integrated with the cell's chromosomal structure.[3] Other hypotheses (and data to support them) by which nuclear genetic structures or functions may be influenced by "cytoplasmic states" or environmental conditions have been considered by Nanney (21, 22) in his discussion of epigenetic phenomena, and by Schultz (23).

From the biochemist's point of view, the crucial questions are the nature of the chemical constitution of regulating genes, episomes, or epigenetic elements, as well as a more precise definition in biochemical terms of the mechanisms of their functional interaction with the cell's hereditary machinery. Evidence for a genetic function of RNA is now apparent from work with RNA viruses (see 19), and a semi-autonomous role of this nature has been postulated for mammalian cellular RNA; this has been recently discussed in connection with the field of cancer (6, 24). Since it seems unlikely to these re-

[3] The concept has arisen from studies with temperate bacteriophage, and the relevance for viruses is apparent. However, various non-viral examples can also be cited (14), and it is possible, as pointed out by Luria (4, 5), that many types of genetic elements may be transferable from cell to cell by one means or another and that we are merely more aware of those for which assay methods now exist (such as viruses). The restriction of the term "virus" to elements capable of independent intercellular transfer and cell invasion is discussed below.

viewers that RNA of mammalian cells would ever take on an autonomy of replication or function equivalent to that of viral RNA, this question is of only theoretical importance. For carcinogenesis, the major unknown is still the mechanism of initiation.

Immunological aspects.—Immunity is one facet of the host-neoplasm relationship that is attractive and appears to hold promise. The encouraging results of recent years appear to signify, according to some, the probability of the development of useful diagnostic and preventive procedures as in other diseases.

The fundamental problems in this area are far from being solved. Two most important questions to be answered are: (*a*) Do neoplastic tissues possess or lack antigens that are not present in corresponding normal tissues? (*b*) What is the relationship of such modification to the process of carcinogenesis?

Investigations of the role of immunological differences between neoplastic and normal cells have been most definitive when the background of the homograft reaction (resulting from homologous grafting of tissues) and the known genetic and antigenic difference of inbred strains of mice have been utilized. A recent report of Gorer & Mikulska (25), which details the present knowledge of one histocompatibility system of the mouse (H-2), reveals the ever-increasing complexity of this system; more than 20 H-2 components have now been identified. There is less information on the other known histocompatibility loci, H-1 and H-3. These are not as important as H-2 in the homograft reaction, but it is likely that the many minor histocompatibility loci are additive in their effects and may in certain situations reflect strong reactions.

The immune reaction called forth through transplantation of normal or neoplastic tissues is only one of several different kinds of such reactions and is characterized by certain features: (*a*) the reaction is provoked apparently by nuclear antigens; (*b*) it is not necessarily distinguished by the formation of soluble antibodies; and (*c*) it is transferrable only by cells.

As pointed out by Medawar (26), the homograft reaction has become part of the "instrumentation" of cancer research. There is little in the recent literature on changes in the antigenic make-up of transplantable neoplasms to indicate that the immunological characteristics of such neoplasms are not the result of genotype differences between tumor cells and cells of the host. Most of the changes observed can be ascribed to histocompatibility differences of the H-2 or other H-alleles and result either from a mutation-selection process or, in certain rare instances, from host-induced modifications (see 27). It is unlikely that adequate data have been presented thus far to indicate a primary immunological difference between a neoplasm and its normal tissue homologue; however, it is quite clear that a striking characteristic of some, but not all, neoplastic cell populations is their capacity to resist isoimmune responses (see 28). The antigenic changes observed in neo-

plastic tissues following the use of other techniques, such as precipitin reaction, hemolysis, complement-fixing reactions, and anaphylaxis, are discussed below.

Recently two lines of evidence have been presented which suggest that antigenic components may be present in neoplastic cells, although they are not demonstrable in their normal counterparts. In extension of the work of several investigators (29 to 31), Prehn & Main (32) studied the immune response evoked by methylcholanthrene-induced sarcomas in certain substrains of inbred mice. It was found that most of the induced sarcomas possessed an isologous antigenicity not shared by spontaneously arising sarcomas or by mammary carcinomas and certain normal tissues. Mice in which the original neoplasms were removed by ligation or by surgical excision were resistant to subsequent inoculation with the same neoplasm, and, interestingly, cross-immunity was demonstrated among different induced neoplasms. That the action of methylcholanthrene in this situation is more than concomitant heritable alterations in antigenicity, unrelated to those changes that result in malignancy, is reflected in a recent report of Prehn (33); this suggests that immunization against (or prevention of) methylcholanthrene-carcinogenesis may be achieved by use of methylcholanthrene-induced sarcomatous tissues as a source of antigen.

Types of antibody, designated anti-X, that are not directed against H-2 or other known histocompatibility loci have been demonstrated by passive immunity studies (34, 35) in certain lymphomas and leukoses of mice and are of interest since they appear to be limited to neoplastic tissues. It appears from the data that circulating antibodies alone are responsible for the cytotoxic or cytostatic effects observed. Much of the earlier work was done with long-transplanted leukoses, and it is of interest to note the presence of such components (antigens) in autochthonous or early transplantable leukoses (36). More recent evidence also points to the probability that leukoses arising in different strains of mice are distinct. Cross reactions were not observed among different X-type antigens from leukoses of the inbred strains DBA/2, A, and C3H (35). On the other hand, a series of leukoses, most of which are of recent origin in the Gorer strain of C57BL mice, appears to be interrelated but not identical (36). The nature of X-components and their significance remain to be determined. It is probable that the X-components represent some substance peculiar to the leukoses and may constitute a mutation (not H-2) in the leukemia cell or antigenic products of virus-like agents that are propagated by the leukemic cell.

The concept that carcinogenesis is initiated by immunological changes, as proposed by Green in 1954 (37), has received further consideration (see 38). Additional evidence has been presented that purports to show that carcinogen-bound protein is specifically antigenic, that antibody to it is produced in the carcinogen-treated animal, and that the local tumor-inhibiting action of certain polycyclic hydrocarbons is caused by induced antigenic change in the cancer cell. It is to be recalled that the immune reaction in-

duced by a carcinogen (loss of specific cellular antigen) was concluded to be the essential cause of neoplasia. The initiating stage involved the alteration of isoantigens, and the resulting antibody response eventually led to a cellular adaptation that involved the loss of identity-protein complex with resulting emergence of the neoplastic cell. Thus the loss of identity-proteins was said to constitute the neoplastic state.

A recent report by Weiler (1) concerning the loss of tissue-specific antigens in 4-dimethyl-aminoazobenzene-induced hepatomas in rats and stilbestrol-induced carcinomas in the hamster and in tissue culture preparations is of interest. Although the approach here is immunological, it is related to the deletion concept of carcinogenesis (*vide infra*). It is presumed by Weiler that loss of immunological character results in proliferative growth because of the loss of proliferation-inhibiting functions. Using liver hepatomas induced with 4-dimethyl-aminoazobenzene, Hughes *et al.* (39) have confirmed the fact that there is loss of an important protein. An important unanswered question, however, as pointed out by Burnet (1), is whether the difference between the proteins concerned is indeed an immunological one.

The role of tumor nucleoproteins in antibody production and antigen specificity has been further studied (40 to 42). As in earlier work, antisera to such preparations have proved to be cytotoxic, but, with the extension of the studies to more tumor lines carried in tissue culture, there has been less apparent specificity of the cytotoxicity for the tumor from which the preparations were derived, and the extent of cytotoxicity has not correlated with the degree of malignancy of the original tissue. Two types of antisera were distinguishable, and sensitivity to each of these correlated with morphological characteristics. Schmidt *et al.* (43) have also discounted the specificity of antisera against tumor deoxynucleoprotein, but they have concluded that a tumor (hepatic carcinoma) nucleoprotein was antigenically deficient in comparison with that from normal tissues, in that γ-globulin antibodies of the tumor antisera failed to concentrate in the tissue of origin; this deficiency was apparently in the protein and not in the DNA component.

Convincing quantitative differences have been observed between the antigenic components of neoplastic and so-called normal control tissues. Several recent claims have been made for qualitative specificity of antigens in animal and human neoplasms; this has been revealed principally through the anaphylactic reaction in guinea pigs (44 to 46). Where claims have been made for specificity, experiments have seldom beeen controlled in a manner that allowed unequivocal identification or isolation of specific neoplastic antigens. It has been shown, for example by Baldwin (47), that striking quantitative differences in hemolytic activity exist between neoplastic and normal tissues.

After discussion with Burnet, Lederberg (48) has reformulated the elective theory of antibody formation (by which an antigen stimulus serves to stimulate a specific, already-existent mechanism) in biochemical genetic terms, which are based on our present state of knowledge of bacterial genet-

ics. According to this, the potential for specific antibody formation by a cell lies in the nucleotide sequence of a particular segment of its chromosomal DNA (gene), and genic diversity of antibody-forming cells is accounted for by a hypermutability of this gene throughout the lifelong proliferation of these cells.

The "Warburg theory."—The Warburg theory of carcinogenesis originally attributed cancer to injury to a cell's respiratory mechanisms with resulting conversion to anerobic metabolism as a means of survival; modern restatements, extensions, and criticisms of this theory have been discussed in various recent reviews (17, 49 to 53). Warburg himself has made further recent extensions (54). He has considered the high glycolysis of non-tumor (kidney) cells grown in tissue culture to be associated with a transformation to malignancy. Manometric and metabolic studies on such cells were reported to reveal that (*a*) glycolysis, uncoupling of respiration, and *in vitro* growth began together, followed by a fall in respiration; (*b*) reduction in catalase was a far more sensitive indicator of the metabolic changes than was reduced respiration; and (*c*) all of these early changes (in glycolysis, respiration, and catalase content) were followed by a long lag phase before transformation to malignancy was accomplished, a phase of development of resistance to the host's "cytolysins," which Warburg calls a "question of immunochemistry." Thus, autonomous malignant growth is apparently explained on the basis of immunological phenomena as well as of alterations in carbohydrate metabolism.

Chemical carcinogenesis.—This subject was emphasized in last year's review (3) and will be dealt with only briefly here. The important observations of the Millers (55), which show a binding of the hepatocarcinogenic aminoazo dyes to liver proteins, have been extended to other tissues and to other carcinogens. Correlation of the phenomenon with aminoazo dye carcinogenesis has been made by the Millers; a recent review by Heidelberger (1) covers pertinent findings and their bearing on the deletion hypothesis of carcinogenesis. More recent work by Heidelberger & Davenport (56) has dealt with attempts to fractionate and characterize the skin proteins with which carcinogenic hydrocarbons interact. Electropherograms of the water-soluble skin proteins indicated the probability of a specificity of the proteins to which the carcinogen, 1,2,5,6,-dibenzanthracene (but not 1,2,3,4-dibenzanthracene), was bound. It was pointed out that, interestingly, the electrophoretic "h" component of liver proteins, to which aminoazo dyes are principally bound, migrated to the same region as the sensitive skin proteins in column electrophoresis. This paper (56) also reports an interaction of hydrocarbons with the nucleic acids of mouse skin. Through the use of radioactive dibenzanthracene of high specific activity, evidence of binding to both RNA and DNA was found, and the specific activity was considerably higher than that of the most radioactive protein fraction obtained. The firm binding here was presumably to oligonucleotides. The significance, in terms of carcinogenesis, of the nucleic acid binding, as well as that to proteins, depends upon a fuller

characterization of the specific proteins and specific nucleic acid sites involved.

A problem in the "deletion" concept is the difficulty of seeing clearly how a specific enzyme (protein) deletion that results from binding may be reflected in the DNA of the somatic cell genome in such a way as to produce heritable, irreversible, and infiltrative growth. The possibilities have been discussed by Potter (17) and are considered above (p. 578). Observations such as those recorded by Heidelberger & Davenport (56) on nucleic acid binding could make possible more easily tenable hypotheses as to the direct deletion of nucleic acid templates being primarily responsible for the "persistent hyperplasia of cancer."

Interest in the subject of the role of metals and metalloids in the carcinogenic process has been revived by the recent observations of Richmond (57). An iron-dextran complex was shown to produce in rats a frequency of nearly 50 per cent sarcomas at the site of injection; these were observed to appear at from 9 to 14 months of age. The carcinogenic potential in the mouse has been further investigated by Haddow (1), who found that, following repeated weekly injections of the complex, similar neoplasms appeared after a somewhat longer latent period. The possibility exists that tumor induction is not confined to the site of injection and that other morphological forms of neoplasms in other tissues result directly from the influence of the iron-dextran complex. Preliminary evidence points to the carcinogenic action as being a function of iron and not of dextran or of the complex.

The non-specificity of carcinogenic materials is apparent from many data. In this connection it has been pointed out by Walpole (1) that the ease with which sarcomas are induced in connective tissues, particularly of rats, and the number and variety of the substances by which this is effected are remarkable. A similar situation may prevail in the mouse; introduction of extracts of human leukemic tissues and sarcomas (58), of smallpox virus (59), or of a carcinogenic hydrocarbon (60) into neonatal mice has been found to lead to leukemia, while introduction of materials from human neoplasms (61) or from parotid tumors (62) into other mouse strains has produced mammary tumors. The essential functions of these materials, whether of initiating, or promoting, or both of these, should be determined.

The two-stage concept of carcinogenesis is widely recognized as being applicable to certain experimental situations. The range of tissues and species and of "initiators" and "promoters" is, however, extremely narrow, and the mechanism has not been delineated. An interesting recent study of skin carcinogenesis in mice by Cowen (63) has attempted to answer some of the questions concerned with the manifestation of "initiation" and "promotion," particularly the part played by genetically determined differences in susceptibility to carcinogenesis. Using a sensitive strain of mouse 101, a resistant strain CBA, and the F_1 hybrid of these two strains, it was concluded that the condition of "initiation" is localized in the skin and is transferred with it in skin grafting and that the genetically controlled sensitivity to tumor in-

duction with 9,10-dimethyl-1,2-benzanthracene (initiation followed by promotion) is also a local property of the skin. It was pointed out that the results obtained are not relevant to the question brought forward earlier by Marchant & Orr (64) concerning the primary site (dermis or epidermis) at which carcinogenic stimulation occurs within the skin, since full thickness skin grafting was accomplished by Cowen. It seems clearly desirable to graft reactive skin on a non-reactive strain, and vice versa, in order to obtain a definitive answer to the question. The use of 9,10-dimethyl-1,2-benzanthracene susceptible and resistant strains within the same H-2 group, made tolerant by the Medawar technique, would be worth trying.

"*Viral carcinogenesis.*"—There has been a marked revival of interest in the past few years in viruses and cancer (3, 5, 65 to 73); the possibilities have seemed intellectually attractive and, to some, hopeful from the standpoint of control. Granting that carcinogenesis involves "some effect on the cell's heredity mechanisms" (*vide supra*), the problem is not whether a hereditary effect could be mediated by a virus; examples of this exist, especially among bacterial viruses, and the narrowing of the gap between infection and heredity has been repeatedly pointed out [Luria (4, 5); Wollman & Jacob (6); (67)]. The question is, rather, one of mechanism: to what extent does a tumor virus impose genetic information by integration with the cell genome, perhaps in an "episomic" manner, or to what extent is it a completely autonomous replicating unit, acting primarily by an effect on the cell's metabolism? Related to this is the matter of transmission under natural conditions; Luria (5) has restricted the term "viruses" to "elements of genetic material of cells, which . . . can determine the biosynthesis of a specific apparatus for their own transfer to other cells" (cf. 74); this process is still unexplored for many of the tumor viruses. Similarities between tumor viruses and clearly "infectious" viruses have been emphasized by some (72); others have completely avoided the distinction by simply extending the definition of "virus-like" to nearly all "hereditary determinants" [Stanley (65)]. But regardless of verbal definition, experimentally the exploration of mechanisms will depend upon exact genetic analysis with adequate stable markers, and upon studies on the viral control of enzyme and other syntheses, as recently investigated with bacteriophage (19, 75 to 77). Dulbecco (73) has considered that a tumor virus may well be both "integrative" and autonomous, depending, perhaps, upon virus concentration and upon various intracellular and environmental factors that affect host cell conditioning. This last may be especially important for tumor viruses (69, 72 ,78); the nature of the factors involved needs much further exploration, but certainly "competence" of the receptor cell is at least as critical as that of the virus in determining the interaction.

The question next arises as to the essential nature of the viral genetic information in the transformation of a normal cell to one that is rapidly proliferating and malignant. The process has been conceived by some as being basically immunological; recognized viruses are known to affect antigenic properties of the cells in which they reside (67, 79), and this can further

affect, for example, the agglutinating properties of the cells [Luria (5)]. Harris (68) has summarized the evidence that a tumor virus may be simply an antigen-bearing particle capable of interfering with the immunity-producing system. The answer to this question also awaits experimental examination of specific genetic and metabolic interactions between the virus and the host cell.

Tumor Viruses

Rous sarcoma virus.—Recent studies on purified preparations of Rous sarcoma virus have correlated the particle seen under the electron microscope with the tumor-producing activity (80, 81). Bather (82, 83) has, by chemical means, shown that partially purified preparations contained RNA but not DNA; by use of staining techniques and electron microscopy, Epstein & Holt have also demonstrated the presence of RNA and its removal by ribonuclease (84, 85; see also 159).

Improved methods have been developed for assay, *in vitro*, of the virus on chorioallantoic membrane and in tissue culture (86 to 89), and these, plus modern cloning techniques, have made possible a fine series of experiments by Rubin & Temin (87, 90 to 92) on aspects of the virus-cell relationship. Rubin has concluded from his data that cells infected with Rous sarcoma virus can multiply and transmit to their progeny the ability to produce virus without need for reinfection, a situation that, to many observers, resembles the lysogenic state in bacteria. The resemblance of Rous sarcoma virus to temperate phage [cf. Luria (5)] is reinforced by data on the resistance of the free virus to ultraviolet radiation and the contrasting radiation sensitivity of the cell's capacity to support virus multiplication. The latter results have been interpreted as an indication of a common radiation-sensitive target, implying integration of the virus components with those of the host cell for virus replication. However, infected cells can make and liberate low yields of mature virus and also continue to divide, as clearly demonstrated by single cell experiments (91, 92). Hence, if association with the host cell genome controls replication, the mechanism of disassociation must be different from that in lysogenic bacteria, lacking the sudden irreversible shift from provirus to vegetative virus.

Avian leukoses viruses.—The viruses of avian myeloblastosis (93) and of avian lymphomatosis (94, 95) have recently been successfully cultivated in tissue culture. Purified preparations of myeloblastosis virus have been chemically characterized as containing about 2 per cent RNA; no DNA was detectable (96). However, cytochemical studies on the effect of lymphomatosis virus on cells in culture (97) have indicated that intranuclear inclusions which were rich in DNA resulted.

Mouse mammary tumor agent.—The various virus-like particles found in the cytoplasm of mammary tumor cells have been examined by biophysical measurements (98), and it has been concluded that two different-sized particles correspond to the complete virus (100 mμ) and its inner core (20 to 30

mμ), respectively; the presence of an inhibitor (40 to 60 mμ) was also thought to be indicated. Partially purified preparations of the virus have been analyzed chemically and found to contain RNA but no detectable DNA (99; cf. 100). Heston (6) has reviewed the evidence that several genes are involved in susceptibility to the virus. Bittner *et al.* (101) have studied antigenic changes induced by passage in hosts of different genetic make-up; they have postulated that such changes involve incorporation of host tissue components into the virus particle. Tjio & Östergren (102) have indicated that an abnormal heteropycnotic chromosome may be generally associated with virus-carrying mammary tumors; however, the relation to the virus is not clear since a similar chromosome has also been observed in mammary tumor tissue from a virus-free source as well as in certain transplantable lymphosarcomas, in normal tissues, and in regenerating female rat liver (103).

"*Polyoma*" (*parotid tumor*) *virus.*—Present knowledge about the "polyoma" virus has been recently reviewed by Stewart (104). Originally found in leukemic mouse tissue extracts and propagated in tissue culture, it induces, upon injection into newborn mice, tumors of the parotid gland and other organs; the tumors are chiefly of epithelial origin. It has been termed "polyoma" virus because of the multiplicity of tumor types produced by what is apparently a single agent [Furth (1); (105)]. The pathology induced in mice has been thoroughly examined by Dawe, Law & Dunn (106), and the agent's range of attack has been defined with regard to tissue and also mouse strain, age, and litter. The agent has also been found to induce tumors in hamsters (107, 108) and in rats (109); the tumors produced, however, differed for the various species. The agent did not seem to lose activity for any of the three species by passage in another if it was, in the interim, passed again in tissue culture.

Cytopathogenicity of the virus in culture (105, 106, 110 to 113) has been applied to plaque methods of quantitation (110); specificity of the agent was indicated by antiserum neutralization of plaque-forming capacity (110, 111) and by tumor production (110), but quantitation *in vivo* has not yet been achieved. Other new assay methods have utilized red blood cell hemagglutination (105, 112), complement fixation (112), and antibody production (112). The erythrocyte receptor site could be blocked by influenza virus and was similarly destroyed by *Vibrio cholera* receptor-destroying enzyme (114).

Spontaneous "infection" (presence of antibody) was found to be spread slowly among adult mice by room or cage contact (105, 115 to 117); this is not surprising since the agent is excreted in high concentrations in the urine and saliva and is airborne (110, 115). Antibody persistence was high over the time periods studied. Cross-infection is therefore to be expected in laboratories working with the agent; the fact that the virus can apparently also infect other tumors (119) makes this especially relevant. Extracts of other neoplasms [e.g., mouse mammary tumors (119), and neoplastic tissue of human Hodgkin's disease (120)], have been reported to have tumor-inducing properties similar to those of polyoma virus, and the question is whether these

are caused by new, possibly related, agents or merely by the presence in these other tissues of polyoma as an unrelated contaminant. Specific methods for identification of polyoma make it possible to answer this question, and these methods should be applied to materials that induce such a similar spectrum of tumors. In contrast to this spread of virus, tumors were induced only by the introduction of high concentrations of virus to very young mice.[4] The age factor in susceptibility to tumor induction may depend upon inability to develop active immunity with sufficient rapidity; newborn mice inoculated with virus developed antibodies within two weeks, but these did not prevent tumor development (116). However, the resistance of certain newborn litters of both mice (121) and hamsters (122) to tumor induction could be correlated with the presence of maternal antibodies.

The virus was found to be remarkably stable to physical and chemical agents (105, 123, 124), including freezing or lyophilization, ultraviolet irradiation, and a large number of common disinfectants. It was inactivated by a mixture of 95 per cent ethanol and iodine or by heating for 30 min. at 70°C. These results were based on assays, *in vitro;* this heat treatment did not destroy all tumor-producing capacity (105). Mixed nucleic acid preparations from leukemic tissues were reported by Latarjet *et al.* (125) to induce some parotid gland tumors and others; in later work this activity was attributed to DNA and was reported to be destroyed by deoxyribonuclease [Latarjet (73)]. A preliminary paper by DiMayorca *et al.* (126) has also reported infectivity (cytopathogenicity in tissue culture and tumor induction in hamsters) in nucleic acid preparations from tissue culture material and has stated that this activity was abolished by deoxyribonuclease but not by ribonuclease. Buffett *et al.* (127) reported what they considered to be an unusual amount of DNA in their polyoma preparations from cytoplasm. However, Dmochowski and co-workers (128) have reported that RNA preparations from AK mouse leukemic tissues produced cytopathogenicity in tissue culture and that these cultures induced parotid tumors in AKR mice; the cytopathogenic effect of these RNA preparations was removed by ribonuclease. The discrepancies in these results remain to be explained.

Under the electron microscope the partially purified virus appeared as a spherical particle, 40 to 45 mμ in diameter (129). Fluorescent antibody staining (130) and electron microscopy (131) of infected cells showed that the virus was localized or appeared first in the nuclei [see Dulbecco (73)]. Cytochemical studies (113) have also indicated initial enlargement of the nucleolus and alterations in the nucleus, particularly in the normal ribonucleoprotein; no evidence could be found for the formation of new Feulgen-positive material, but the amount or kind of DNA in the virus may not be detectable by current staining techniques.

[4] This has caused some observers to postulate that the role of the virus is indirect [Luria (5)] or that tumor induction is a laboratory artifact [Burnet (5)] and would probably not be mediated by the virus under natural conditions.

Polyoma and leukemia.—Polyoma virus has frequently been found in extracts of leukemic mouse tissues, particularly from AKR mice, and the question has arisen as to a possible relation of polyoma to a leukemogenic agent. Recent studies by Law *et al.* (118) have shown no correlation in various mouse strains (AKR, C58, and C3Hf/Bi) between ease of infection with polyoma (antibody development) and potentiality to develop lymphocytic leukemia, either spontaneously or in response to x-irradiation. Polyoma infection was not particularly strain-specific but, instead, largely dependent on environmental exposure (cf. 116), while leukemia incidence remained characteristic for each strain, in various colonies, regardless of exposure to polyoma. Also, leukemic extracts from AKR or other mice that had been shown by assays, *in vitro*, to be free of polyoma have not been found to induce parotid tumors. The repeated association of the two agents is then apparently fortuitous and probably occurs by cross-contamination of leukemic extracts during their preparation in laboratories in which polyoma is carried (cf. 119, 120). Expression of either leukemogenic or "polyoma" activity in such preparations [e.g. Latarjet (1)] would then be explained by preferential selection for the isolation or replication of one or the other of the agents.

Leukemogenic agents.—An apparent plurality of cell-free agents has been reported to be able to induce, or in some instances accelerate, the development of certain types of mouse leukemia; these various agents have been classified by Furth (1). Gross has compared the agent that induces one type of lymphocytic leukemia [Gross (65, 66)] to a temperate bacteriophage (132); induction of lymphocytic leukemia of thymic origin by filtrates of radiation-induced (as well as spontaneous) leukemia [133, 134; Upton (1)] has also been interpreted as indicating that x-rays may activate a latent virus or other "subcellular genetic determinant" (134).

Evidence against this concept of leukemogenic agents has been reviewed by Latarjet (73). The relationship of the viral agent to the spontaneous appearance of this disease remains unclarified [Law (5)], but, in certain strains of mice, development of filtrate-induced leukemia, like that of spontaneous lymphocytic leukemia, was inhibited by thymectomy (135, 136). Latarjet (1) has reported this agent to be stable to proteases (trypsin and chymotrypsin) and to deoxyribonuclease, alone or in combination; results with these proteases plus ribonuclease were inconclusive. This would eliminate free DNA, but not necessarily deoxyribo- or ribo-nucleoprotein, as the essential constituent.[5]

Further studies have been reported on leukemia induction with filtrates of mesenchymal tumors. Graffi (138 to 140) has reviewed findings on the agent from certain sarcomas and carcinomas that induces myeloid leukemia (and chloroleukemia); it would seem that the agent has a nucleoprotein and

[5] Another preliminary report has appeared (137) that purports to show induction of leukemia in certain mouse strains with purified nucleic acid preparations; presentation of the paper was withdrawn and details of the work are not yet published.

lipoprotein nature (140 to 142). If the "virulence" of the preparations was high enough, some myeloid leukemia was produced by administration to adult mice and to newborn rats; agent passed in rats did not lose leukemogenic activity for mice (143). Filtrates of the induced myeloid leukemia also produced cytopathogenicity in tissue culture, and filtrates from the cultures produced early multiple sarcomas in rats (144). Schmidt (145, 146) has further characterized the similar or identical agent in mouse Ehrlich carcinoma filtrates which induces myeloid leukemia. Moloney (147) has recently reported the induction of mouse lymphocytic leukemia with extracts of mouse sarcoma 37; no other tumors were observed. This agent showed less strain and age specificity than certain other mouse tumor viruses.

Friend (148, 149) has studied further an agent from Ehrlich ascites filtrates that is reported to induce in Swiss mice a reticulum cell neoplasm of unusual morphologic form; the leukemic nature of this lesion, however, has been questioned (150, 151). This agent showed no immunological relationship to those from a number of other mouse leukemias (149). Studies on early biochemical changes in spleen after infection with the Friend agent (152) have been interpreted as indicating biosynthesis of new RNA.

Schwartz & Schoolman have reviewed their data on leukemia development in AKR or Swiss mice following injection of brain filtrates from leukemic mice or human patients (153); comparable data have now been reported for C3H mouse leukemia (154). Filtrates of leukemic tissue itself have been reported to be inactive and to inactivate brain filtrates. Activity has also been claimed in brain filtrates from non-susceptible mice injected with leukemic cells, even though the neoplasm itself could not grow in these mice. These workers have postulated that a specific, heat-labile agent produces an immunological host response, detectable especially in brain tissue, and that leukemia development constitutes an expression of this host response.

Extracts of human neoplasms, especially leukemia, have been reported to reveal virus-like particles (71, 155, 156) and to produce cytopathogenicity in tissue culture (155, 156) and tumor induction in animals (61, 120, 157, 158). The RNA in such preparations has been implicated (156 to 158); two preliminary reports (157, 158) have claimed tumor induction with RNA preparations, but an essential role of the RNA or even of the neoplastic source was not clearly established.

Rabbit papilloma, fibroma, and myxoma viruses.—The reader is referred to other recent reviews (3, 53, 78, 159 to 161) for discussion of these viruses.

Anticancer Agents

Anticancer agents reviewed here include only antimetabolites; these have also been discussed in other recent reviews (50, 162 to 166). Mechanisms of action of these agents are considered here together with mechanisms of resistance to them, since recent work on the latter has provided major clues to the former. It has been especially satisfying to those genetically and biochemically oriented to see that many reports have now correlated heritable

markers of resistance with alterations in enzymatic activities, especially with loss of capacity to convert an analogue to its nucleotide. Biochemical mechanisms of the genetic events themselves remain to be explored, as do the qualitative and quantitative enzymatic expressions of the various "steps" in the mutation process, but the way is now open to specific approaches in these directions.

6-Mercaptopurine.—Mercaptopurine ribotide has been characterized and its biosynthesis described (167, 168). Tumor cells apparently have a high capacity for conversion of the base to the ribotide (167, 168)—perhaps a reflection of the generally high anabolism and low catabolism of purines and pyrimidines in tumor tissues (17, 50, 169). Reduced capacity for this conversion was associated with resistance to the compound in bacteria (170 to 172), leukemia L1210 (168, 170, 173), Ehrlich ascites carcinoma (167, 174), and in mouse fibroblasts in tissue culture (175, 176). Brockman and associates (168, 170, 173) have translated this observation to the enzyme level by showing that extracts of resistant cells (*Streptococcus faecalis* and L1210 leukemia) were deficient in the nucleotide pyrophosphorylase activity that catalyzes the reaction of 5-phosphoribose 1-pyrophosphate with mercaptopurine, hypoxanthine, guanine, and azaguanine; the further anabolism of these bases was also deficient in the resistant lines, thus indicating the critical nature of this initial reaction. Pyrophosphorylases for the conversions of adenine and xanthine to ribotides and the further anabolism of these bases were unaffected. A decreased reaction of phosphoribose pyrophosphate with hypoxanthine, but not with adenine, has also been more recently observed in extracts of mercaptopurine-resistant fibroblasts (176); other results (172, 174, 175, 177) likewise showed decreased formation of these purine nucleotides. Extracts from resistant cells did not inhibit those from sensitive cells (176, 178), and heat-denatured extracts from sensitive cells did not activate preparations from the resistant line (178), so the lack of activity in resistant cells appears to be a simple loss.

Guanine, hypoxanthine, and mercaptopurine have been reported to be converted to ribotides by the same enzyme (179 to 181); partially purified pyrophosphorylase from *Escherichia coli* had an affinity for mercaptopurine about equal to that for hypoxanthine and greater than that for guanine (181), thus indicating an effective inhibition by mercaptopurine (cf. 50); these preparations were reported to be inactive for azaguanine (180, 181). Way & Parks (182), however, reported that a hog liver preparation was active for guanine and azaguanine but not for mercaptopurine; the activity for azaguanine was considerably less than that for guanine and showed a somewhat different pH optimum.

In the cases of resistance to mercaptopurine and azaguanine so far examined (168, 170, 173, 183), pyrophosphorylase activities for hypoxanthine, mercaptopurine, guanine, and azaguanine were lost together. It is, therefore, not clear whether all four conversions are mediated by the same enzyme with activity for some bases not detected in certain systems, whether they are

mediated by two or more enzymes controlled perhaps by a single or by closely linked genes, or whether there are differences in specificity that depend simply upon tissue source of the enzymes.[6] Most of the resistant strains of *Streptococcus faecalis* were unable to utilize purine bases other than xanthine (184, 185),[7] but they metabolized xanthine and were sensitive to azaxanthine (183). One line, which showed cross-resistance to azaxanthine, failed to metabolize xanthine and azaxanthine as well as the other purines (183), and another line which was resistant to azaadenine metabolized xanthine but not adenine (171); AMP and XMP pyrophosphorylases are, therefore, apparently separate enzymatic activities.

Reaction with phosphoribose pyrophosphate is probably the major pathway for purine anabolism [Kornberg (11)], and the purine analogues represent no exception; ribose nucleosides might then arise largely by breakdown of the ribotides. This, plus the rapid breakdown of the nucleoside to the base, could explain comparable antimetabolite activity of a base and its nucleoside and cross-resistance between them (175, 187), and is supported by the relative absence of the ribose nucleosides in resistant cells unable to form the ribotides with phosphoribose pyrophosphate (173, 183). Mercaptopurine nucleoside, however, was reported to be produced by resistant cells lacking this conversion (175), and present data cannot determine whether anabolism goes completely by the pyrophosphorylase pathway, known to be reduced in some resistant lines, or whether an additional pathway by way of nucleoside phosphorylase and nucleoside kinase may also be important, with cross-resistance to the nucleoside implying reduced kinase activity as well; these questions will require exploration at the enzymatic level.

Antimetabolite activity of mercaptopurine thus appears to depend upon the "lethal synthesis" (173, 188) of the ribotide. In recent studies with S^{35} and C^{14} labeling (168, 189), the analogue was apparently not incorporated into nucleic acids except by desulfurization to normal purines, and activity at the nucleotide level seems more likely (cf. 50, 165, 166). In L1210 leukemic cells the drug inhibited incorporation of both glycine and hypoxanthine into nucleic acid adenine (but not guanine), while in resistant cells glycine incorporation was unaffected and hypoxanthine incorporation was

[6] Another mercaptopurine-resistant mutant of *Streptococcus faecalis* (SF/MPcc) appeared to have an alteration in enzyme affinity rather than a simple loss, since pyrophosphorylase activity for azaguanine and mercaptopurine was reduced considerably more than that for hypoxanthine or guanine (170). Multiple mechanisms of resistance apparently exist at least in the bacterial strains (183 to 185) and probably in other systems. Balis *et al.* (186) have concluded that still other mechanisms of resistance in *S. faecalis* depend in one strain upon greater capacity for *de novo* purine biosynthesis and in others upon more desulfurization of the drug to normal purines.

[7] Although the conversion of adenine to AMP proceeded normally (170, 183), there was partial impairment in the conversion of AMP to GMP (185), which probably accounted for inability to grow on adenine. Mercaptopurine-resistant cells in culture have also been shown to be unable to utilize hypoxanthine (175, 176).

more strongly inhibited (177). It was postulated that mercaptopurine ribotide blocked conversion of IMP to AMP (see 50, 166) and that, in resistant cells, the pyrophosphorylase was limiting and not enough mercaptopurine ribotide was formed to exert the block. Hakala & Nichol (190) also concluded that, in tumor cells in culture, mercaptopurine inhibited conversion of IMP to AMP; the effect was reversed competitively by IMP and non-competitively by AMP. In work with cell-free preparations, Salser *et al.* (172) found that conversion of IMP to adenylosuccinic acid, and thus AMP, in *Streptococcus faecalis* preparations and of IMP to XMP, and thus GMP, in pigeon liver extracts were both inhibited by mercaptopurine ribotide; conditions and accuracy of the assays did not, however, permit quantitative comparisons of the two sites of inhibition. Hampton *et al.* (191) found that mercaptopurine ribotide, as well as the 6-mercapto analogue of adenylosuccinic acid, inhibited cleavage of adenylosuccinate by purified yeast adenylosuccinase; the latter analogue also inhibited this enzyme in its cleavage of 5-aminoimidazole-N-succinocarboxamide ribotide (192). Mercaptoadenylosuccinate was itself cleaved by the enzyme to mercaptopurine ribotide (191), but its effect was greater than could be accounted for by this conversion and, hence, was apparently independent (193). Tomizawa & Aronow (175) have postulated a specific effect of mercaptopurine on DNA synthesis, since toxic effects of even brief exposure to the drug were noted several cell divisions after removal of the analogue. However, no preferential effects on DNA synthesis were apparent in isotope incorporation experiments (194).

Way *et al.* (195) reported indirect evidence that mercaptopurine ribotide could be further phosphorylated by mammalian tissue preparations. Xanthine oxidase catabolized mercaptopurine to thioxanthine and thiouric acid, which were inactive as antimetabolites (165, 189, 196). Skipper *et al.* (187) have investigated structure-activity relationships and cross-resistance patterns for a series of derivatives of mercaptopurine, thioguanine, and chloropurine. Various carcinostatic antimetabolites, including mercaptopurine, have been shown to affect immunological response, suppressing antibody formation and homograft reaction (197 to 201); the possible relation of these effects to anticancer activity is of considerable interest (see 202).

8-Azaguanine.—Azaguanine is incorporated into nucleic acids, especially RNA, by replacement of guanine (50, 162, 164 to 166) and might thus produce an abnormal or non-functional RNA containing an altered nucleotide sequence. Infectivity of certain RNA viruses was decreased after azaguanine incorporation (162); studies have not been made on infectivity of analogue-containing isolated RNA. In living cells, abnormal RNA might disturb protein synthesis; specific enzyme activity (penicillinase) was more sensitive to the drug than was amino acid incorporation into protein, suggesting, perhaps, an effect on amino acid sequence (and RNA template) (203, 204). Inhibition of enzyme synthesis has also been reported by others (165, 205). In *Bacillus cereus*, azaguanine stimulated the synthesis of new RNA coincident with an inhibition of protein, but not DNA or cell wall biosynthesis (206,

207); the new RNA, which incorporated analogue in a labile position (206), appeared to have a high turnover and was postulated to be specifically involved in protein synthesis (206; cf. 208 to 210). Inhibition of protein synthesis has similarly been attributed to thiouracil incorporation into RNA (210, 211). Azaguanine inhibition of antibody synthesis (199) has been mentioned above.

Azaguanine might also inhibit protein synthesis as a fraudulent guanosine triphosphate coenzyme for amino acid activation (see 165, 203 to 205), a hypothesis which could now be tested *in vitro*. Azaguanine yielded azaguanylic acid with nucleotide pyrophosphorylase [(182); *vide supra*]; it was also further anabolized to higher phosphates (183, 195), and the triphosphate was isolated and characterized (195). The analogue might exert both a rapid effect at the nucleotide level and a more delayed one resulting from abnormal protein synthesis on an abnormal RNA template (cf. 204, 212).

Azaguanine affected incorporation of amino acids into bacterial protein in different ways (207, 212; cf. 164, 203 to 206); incorporation of sulfur-containing amino acids was preferentially inhibited (212), while incorporation of glycine, glutamic acid, and especially alanine was increased (207). Synthesis of the cell wall precursors, glucosamine and diaminopimelic acid, was also increased; and incorporation into these compounds produced fortuitously high figures for serine and aspartic acid uptake (207). Azaguanine-inhibited *Bacillus cereus* thus contained excess RNA (206) and cell wall material (207) and reduced protein, a quantity of which differed in composition from that of the normal cell (207).

Initial conversion to the ribotide appeared critical for any antimetabolite activity, since reduced capacity for this conversion was associated with resistance to the drug in *Streptococcus faecalis* (170, 183) and in leukemia L1210 (170, 173). As with mercaptopurine (*vide supra*), extracts of resistant cells were deficient in catalyzing the reaction of phosphoribose pyrophosphate with azaguanine, guanine, hypoxanthine, or mercaptopurine to form the respective ribotides (170, 173, 183). Sensitive cells also converted azaguanine to di- and triphosphates and incorporated azaguanylic acid into nucleic acids, whereas resistant cells failed to carry out any of this anabolism. Tumors with a "natural" resistance to azaguanine were also low in the capacity to anabolize the base (173). In L1210 leukemic cells, the possibility of a difference between the sensitive and resistant lines in permeability to azaguanine has been excluded (213).

Azaguanine resistance has also been studied in human bone marrow cells (214, 215) and in mouse fibroblasts (216) cultured *in vitro*. In these systems, a high level of resistance arose, apparently as a single-step mutation, in contrast to stepwise emergence of azaguanine resistance in L1210 leukemia *in vivo*. As in L1210, the resistant line of bone marrow cells arose by drug selection of mutant(s) pre-existent in the wild type population, but mutation frequency for the marker was high (1 per 1000 sensitive cells) (215).

2,6-Diaminopurine.—Diaminopurine and 2-methyl-diaminopurine (217)

were converted to their respective ribotides by cell suspensions of *E. coli*, and this was inhibited by adenine (218). A resistant mutant was unable to carry out these reactions, although it did convert diaminopurine to its methyl derivative at 60 to 90 per cent of the rate of normal cells, indicating that at least some of the compound entered the cells. Diaminopurine-resistant mouse fibroblasts in culture could use hypoxanthine but not adenine, and extracts of resistant cells caused phosphoribose pyrophosphate to react with hypoxanthine but not with adenine (176); as in mercaptopurine resistance (*vide supra*), this was not referable to inhibitors in resistant cell extracts. A diaminopurine-resistant mutant of *Salmonella typhimurium* also appeared deficient in formation of the ribotide and in incorporation of purines (219); differences in transport concentration of purines were also noted. These findings have suggested identity of the pyrophosphorylases for conversion of adenine and diaminopurine to their ribotides; AMP pyrophosphorylase from yeast, however, was inactive with diaminopurine (220), and the specificity needs further study. Results with bacteria suggest that diaminopurine might compete with hypoxanthine and guanine at the level of nucleoside phosphorylase (221). Indirect evidence has been presented for further phosphorylation of diaminopurine ribotide (195, 218).

6-Thioguanine.—LePage and co-workers found that in Ehrlich ascites cells thioguanine was metabolized to the ribose nucleoside, the ribotide (by reaction with phosphoribose pyrophosphate), probably di- and triphosphates (222), and nucleic acids (223, 224). It was catabolized to thioxanthine and thiouric acid, more so in normal tissues than in tumors (222). A resistant line, *in vivo*, formed somewhat more of these catabolites, and the drug disappeared from the cells even more rapidly than accounted for by this breakdown; differences in catabolism were cited as the major basis of resistance. The resistant line also anabolized less thioguanine to its ribotide and to nucleic acids than did the sensitive line (223), but, since this difference was mostly eliminated by azaserine, the enzymatic mechanisms were apparently not lost. Decreased ability to make the ribotide has been found by others in thioguanine-resistant tumor lines (225, 226); "natural" resistance in tumors was not thought to correlate with this capacity (223, 224) but did correlate with incorporation into nucleic acids (224). From studies with nucleic acid precursors, various metabolic blocks were postulated, including an inhibition of the conversion of IMP to AMP (227). The analogue also inhibited the accumulation of formylglycinamide ribotide, which is mediated by azaserine. Gots and co-workers (228, 229; cf. 230) found that thioguanine, mercaptopurine, and diaminopurine similarly inhibited accumulation of aminoimidazolecarboxamide ribotide in an *E. coli* mutant; they concluded that the analogues mimicked normal purines in inhibiting *de novo* synthesis by a "feedback" mechanism.[8] This could involve effects on enzyme synthesis or ac-

[8] Feedback control was also implicated in the action of a tryptophan analogue, and resistance was thought to involve an altered enzyme insensitive to this control (231).

tivity or could simply be caused by competition, between the analogue and the *de novo* pathway, for phosphoribose pyrophosphate. The latter has been cited as a limiting factor in purine biosynthesis *in vitro* (232; but cf. 233).

Other purine analogues.—(See also 50, 163, 165, 166.) 6-Chloropurine and 6-chlorouric acid were found to inhibit purine-oxidizing enzymes (234). The pyrazolopyrimidines were enzymatically converted to ribotides (182) and probably higher phosphates (195). Unsubstituted purine and its ribose nucleoside were both inactive with nucleoside phosphorylase; the ribotide yielded the diphosphate with adenylate kinase, but this was inactive with polynucleotide phosphorylase from *Azotobacter agilis* (235). Adenine-1-N-oxide was found to act both as an antimetabolite and as an adenine substitute, the latter presumably by reduction to adenine; the oxides of adenosine and AMP were potent inhibitors of sarcoma 180 in tissue culture (236 to 239). A new adenine-containing antibiotic, psicofuranine [6-amino-9-D-psicofuranosylpurine (240)] has been studied by the group at Upjohn Laboratories (241 to 243), and its antitumor properties have been explored (243); one organism, which showed response *in vivo* but not *in vitro* to this antibiotic, developed resistance *in vivo* following exposure *in vitro* (242).

6-Azauracil.—Recent work on azauracil has been reviewed (166, 244, 245). In *E. coli*, the analogue formed the ribose nucleoside, the ribotide, and possibly polyphosphate derivatives; phosphorylation of the nucleoside was apparently mediated by uridine kinase, and competition with uridine at this site was suggested as a point of antimetabolite action (245). In contrast to uridine, azauridine was not degraded, perhaps because of specificity of the nucleoside phosphorylase (245). The accumulation of various nucleic acid precursors (245, 246) could be related to the finding that azauridine diphosphate was an effective inhibitor of *E. coli* polynucleotide phosphorylase (247, 248); the fraudulent diphosphate was itself inactive with the enzyme. Accumulation of N-acetylamino sugar esters was also observed in inhibited *E. coli*, and interference with cell wall biosynthesis was postulated (249); no such effect was noted in *Bacillus cereus* (250).[9] *Streptococcus faecalis* showed a similar anabolism of azauracil (252). A resistant mutant did not metabolize uracil and azauracil but did metabolize uridine and was inhibited by azauridine (253); in contrast to the purine-anabolizing system (*vide supra*), the loss here was presumably nucleoside phosphorylase rather than nucleotide pyrophosphorylase.

In contrast to bacteria, mammalian systems did not readily anabolize the base, but did convert the nucleoside to the ribotide, and azauridine was much more effective than azauracil in these systems (245, 254 to 256). A conversion to polyphosphate anabolites or incorporation into nucleic acids was not detected (252, 254, 256). Azauridine interfered with orotic acid anabolism *in vivo* and *in vitro* (256) and caused accumulation of orotidine (254, 256); this was explained by findings of Pasternak & Handschumacher (256) who

[9] Interference with uridine nucleotide coenzyme function in galactose metabolism was not detected in certain mammalian systems (251).

showed that azauridylic acid was a potent inhibitor of partially purified orotidylate decarboxylase and produced an accumulation of orotidylic acid and hence of orotidine. Since azauridine was without effect on the purified enzyme, its activity in crude preparations and *in vivo* was presumably by conversion to the ribotide. Inhibition of the conversion of orotic acid to uracil was also reported in *Bacillus cereus* (250).

5-Fluoropyrimidines.—Fluorouracil was anabolized in Ehrlich ascites cells to the ribose nucleoside, its mono-, di-, and triphosphates, and to fluorodeoxyuridylic acid (257, 258). The base reacted with uridine phosphorylase, inhibiting a reaction with uracil, and the nucleoside was then phosphorylated with ATP; this appeared to be the major anabolic pathway in mammalian tissues (259). Fluorodeoxyuridine was also converted to the 5′-phosphate; the ribotide formed di- and triphosphates, but fluorodeoxyuridylic acid was apparently not further phosphorylated (260). Fluoroorotic acid was also converted to fluorouridine mono-, di-, and triphosphates (257). The first of these steps occurred with yeast orotidylate pyrophosphorylase by reaction with phosphoribose pyrophosphate; the K_m values for orotate and fluoroorotate were similar, and fluorouracil was inactive (261). Catabolism of fluorouracil resembled that of uracil in that it yielded dihydrofluorouracil, α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine; α-fluoro-β-guanidopropionic acid also appeared to be formed (166, 262). Tumors did not degrade the analogue to any measurable extent. The fluoropyrimidines were incorporated into RNA (but not into DNA) as fluorouracil, apparently by replacement of uracil (257, 263); the deoxyribonucleoside was presumably incorporated by breakdown to fluorouracil (260). The analogue inhibited incorporation of uracil and orotate into RNA (258, 264); fluorodeoxyuridine showed such inhibition only if preincubated with the cells before their exposure to labeled uracil (260).

Fluoropyrimidines, especially fluorodeoxyuridine, markedly inhibited incorporation of formate, orotate, uracil, and deoxyuridine, but not thymidine, into DNA thymine (258, 260, 264, 265). This suggests a specific block in the biosynthesis, but not in the incorporation, of thymidine, most likely a block at the methylation step; indeed, fluorodeoxyuridylic acid was found to be a potent and irreversible inhibitor of thymidylate synthetase in *E. coli* (164, 266). In mammalian cells also, fluoropyrimidines inhibited mitosis or DNA synthesis with results resembling those seen in unbalanced growth and thymineless death (267, 268).

Fluorocytosine had little biological activity, but its ribonucleoside and deoxyribonucleoside had antitumor activity (269) and inhibited growth of mammalian cells in culture (270). Inhibition by fluorodeoxycytidine again suggested a block of thymidine biosynthesis (270), and fluorodeoxycytidylic acid inhibited thymidylate synthetase (266). It has been suggested that these compounds act by conversion to fluorouracil derivatives (260, 266).

As with azauracil and the purine analogues (*vide supra*), resistance to fluorouracil has been associated with inability to anabolize the base. Reichard *et al.* (271) found that extracts of resistant Ehrlich ascites cells were unable to convert uracil to either the ribonucleoside or the deoxyribonucleoside. The

two nucleoside phosphorylases have been partially separated (271), but the relation of the double loss to the genetic events is unexplored.

In further work (272), four other resistant lines of this tumor showed an 85 to 95 per cent decrease in uridine kinase activity but no loss in phosphorylase activities; the kinase loss appeared in a stepwise manner, paralleling the appearance of resistance. Both of these types of enzymatic loss were stable in the absence of drug, as was the resistance. Reduced capacity for uracil or fluorouracil anabolism has also been found in resistant lines of leukemia L1210 (271) and of the mast cell neoplasm P815 (273). In bacteria, uracil anabolism can also occur by reaction with phosphoribose pyrophosphate, and cell-free preparations from a fluorouracil-resistant line of *E. coli* showed a decrease in uridine monophosphate pyrophosphorylase activity; the reaction of phosphoribose pyrophosphate with uracil and fluorouracil was reduced, while that with orotic acid or fluoroorotic acid was unaffected (274).

A different mechanism of resistance in Ehrlich ascites has recently been emphasized by Heidelberger and associates (275, 276). These workers also observed a decreased anabolism of uracil and fluorouracil in the resistant line, but concluded that there was a much greater difference in the extent of analogue inhibition of DNA thymine biosynthesis. Data on formate incorporation into DNA thymine were interpreted as indicating a decreased affinity, in the resistant line, of thymidylate synthetase for fluorodeoxyuridylic acid; the amount of fluorodeoxyuridylate in the resistant cells was the same, but extacts showed less inhibition of the conversion of uridine and formate to thymine derivatives. Lindner (268) also noted that fluoropyrimidine inhibition of DNA synthesis was not evident in resistant cells.

5-Bromouracil and other pyrimidine analogues.—Bromouracil is one of several halogenated pyrimidines that can replace thymine in DNA, with inhibitory and mutagenic effects (162). Induced mutations occurred only at specific locations, which were different from those affected by proflavin-induced or by spontaneous mutations (277); Freese (278) has considered that the analogue-induced mutations might also involve a different type of DNA change than those occurring spontaneously (see also 19). Analogue incorporation produced mutations in DNA in the absence of protein synthesis (during chloramphenicol inhibition) (279).

Bromodeoxyuridine was extensively incorporated into DNA in mammalian cells in culture (280, 281).[10] This incorporation represented a replace-

[10] Such incorporation would depend upon the presence of enzymes able to convert the analogue to the necessary nucleotide precursor of DNA; thus, Bessman *et al.* (282) have shown that, although the deoxyriboside triphosphates (prepared chemically) of both uracil and bromouracil react with the *E. coli* enzyme for DNA polymerization, only bromouracil is incorporated into DNA, apparently because the cells do not contain kinases for conversion of deoxyuridylic acid to the triphosphate derivative (cf. 19). Zamenhof *et al.* (283) concluded that incorporation also depends upon rates or amounts of these necessary conversions, which control the synchronization of nucleotide formation with cell division. They also reported a bromouracil-thymine exchange in DNA, a kind of "DNA turnover" in non-dividing cells that showed no net synthesis of new DNA.

ment of thymine and was more extensive in the presence of amethopterin, which inhibited *de novo* synthesis of thymine (280). After analogue incorporation, DNA and protein were synthesized for a time, and at least one cell division occurred but, eventually, abnormal non-viable cells developed and further DNA synthesis appeared to be prevented (281). The abnormalities did not correspond to those of thymidine deficiency per se, and analogue incorporation was not reversed by thymidine. However, bromodeoxyuridine inhibited incorporation of various precursors into DNA thymine, and this activity was apparently exerted at the stage of incorporation of acid-soluble precursors into DNA (284, 285). Bromodeoxyuridine also inhibited antibody synthesis (*vide supra*, 201).

The antimetabolite activity of thiouracil has been attributed to conversion to the ribonucleoside and incorporation into RNA (162, 210, 211, 286). Thiouracil inhibition of the synthesis of phage proteins was prevented by ultraviolet radiation, which also prevented incorporation of the analogue into RNA (210). Cooper (287) and Jaffe & Cooper (288) have shown that 6-uracil methyl sulfone underwent a non-enzymatic reaction with sulfhydryl compounds by which the methyl sulfone group was replaced by the RS- group of the reacting RSH compound; however, this type of reaction was apparently not responsible for the antitumor activity or toxicity (288). The antipyrimidine activity of urethan and of N-methyl-formamide have been reviewed elsewhere (50, 163), as has the presence of unusual pyrimidine and purine components (5-ribosyluridine, 5-methylcytosine, 6-methylaminopurine, etc.) in RNA (19).

Folic acid analogues.—Aminopterin and amethopterin were potent inhibitors *in vitro* of the reduction of folic acid or dihydrofolic acid, or both, to tetrahydrofolic acid (165, 166, 289 to 291). Wacker *et al.* (292) found that in *Enterococcus stei* aminopterin blocked the conversion of folic acid to the 5-formyl-tetrahydro derivative of a polyglutamyl form; they suggested inhibition of the formylation, but it could apparently as well have been of the reduction since the steps were not separated by the assay methods used. In a resistant strain, aminopterin failed to inhibit this conversion; this did not result from failure to take up the analogue, since unaltered aminopterin was recovered from extracts of resistant cells in larger quantities than from sensitive cells; the authors concluded that the resistance mechanism was one of altered affinity of the sensitive enzyme(s) for the analogue. Similar conclusions had been reached in previous studies on sulfonamide resistance (293). Greater uptake of aminopterin by resistant cells has also now been reported in *Streptococcus faecalis* (294), with the similar conclusion that, although the analogue could be recovered apparently unaltered from resistant cells, it must in some way in these cells be rendered innocuous or its access to the sensitive enzyme system(s) prevented. Resistant cells also took up more folic acid and showed greater conversion of folic acid to 5-formyltetrahydrofolic acid. Capacity for this conversion appeared to increase with the level of resistance, and inhibition of the conversion by amethopterin decreased; this could not apparently be explained only by a difference in

enzyme affinity, since the change with level of resistance was much less apparent with extracts than with intact cells. Since the pteroic acid derivative of amethopterin, to which the strain was not cross-resistant, was much more potent than the other analogues in inhibiting this conversion in whole cells, but not in extracts, it was suggested that the "binding" that renders the other analogues ineffective in intact cells might involve the glutamic acid portion of the molecule.

Biochemical mechanisms of resistance to amethopterin have also been studied in mammalian cells (166, 295 to 297). Based on reversal data, Aronow (295) concluded that in mouse fibroblasts in culture the site of amethopterin inhibition was at some step in tetrahydrofolic acid utilization rather than in its biosynthesis; conversion of aminoimidazolecarboxamide ribotide to IMP was suggested as the sensitive step, as also indicated by data on inhibition in L1210 leukemia (296). There was no indication of folic acid-independent metabolism (295), but in resistant lines of two tumors (296, 297) *de novo* purine synthesis continued in the presence of the analogue and therefore appeared to be insensitive to the inhibition. Biesele *et al.* (6) reported some correlation of amethopterin resistance with chromosomal abnormalities.

Alteration of the homograft reaction by amethopterin has been mentioned above (200; cf. 166, 298). As reviewed elsewhere (163, 166), certain halogenated derivatives of amethopterin have recently been reported to be effective anticancer agents.

Azaserine and 6-diazo-5-oxo-L-norleucine.—Azaserine and 6-diazo-5-oxo-L-norleucine effectively inhibited the reaction of formylglycinamide ribotide with glutamine to form formylglycinamidine ribotide in cell-free preparations of avian liver [164; Buchanan (299)] and of a mammalian tumor (300); azaserine inhibited this reaction in extracts of *E. coli* as well (301). This inhibition has been further studied with radioactive azaserine and highly purified amidotransferase from chicken liver (302). The binding of azaserine to the enzyme was highly specific; both the binding and the enzymatic reaction were inhibited by *p*-chloromercuribenzoate, and the addition of substrates decreased this inhibition. Azaserine and diazo-oxo-norleucine also inhibit other glutamine-requiring aminations. In contrast to formylglycinamide ribotide amidination, azaserine had a much lower affinity than did glutamine for a calf thymus enzyme that catalyzed the amination of XMP to GMP (303). In cell suspensions (304) and soluble preparations (305) of Novikoff hepatoma, both analogues, but especially diazo-oxo-norleucine, inhibited the conversion of orotic acid to cytidine nucleotides, presumably the glutamine-requiring amination of uridine nucleotides. In an extract of an *E. coli* mutant, both analogues inhibited the formation of anthranilic acid from shikimic acid-5-phosphate and glutamine (306). With purified diphosphopyridine nucleotide synthetase from yeast, azaserine was an effective inhibitor of the amination of nicotinic acid-adenine dinucleotide with either glutamine or ammonia as amide donor; these two donors could apparently be utilized independently by the same enzyme (307).

Azaserine inhibition of *E. coli* growth was reversed by aromatic amino acids, and recent data have indicated that in this organism azaserine inhibition of *de novo* purine synthesis in cell suspensions (301, 308), and specifically of formylglycinamide ribotide amidination in extracts (301), could also be reversed by aromatic amino acids. The mechanism is not clear; amino acids might decrease entry of the analogue into the cell (308; cf. 309), but this explanation would seem to be ruled out by the fact that reversal was also apparent in extracts and affected the amidination specifically. It was suggested that the amino acids might somehow reduce affinity of the sensitive enzyme for the inhibitor (301). Inactivation of azaserine by tissue enzymes has been discussed by Reilly (299); the initial reaction is apparently a deamination, after which the diazo keto acid is thought to break down further spontaneously. Of various amino acids tested, the reaction was specific only for azaserine and for acetyl-L-serine, and was not observed with diazo-oxo-norleucine.

Azaserine resistance in TA3 carcinoma was attributed to a greater ability of resistant cells to utilize preformed purines and to recover capacity for *de novo* synthesis after exposure to the antagonist (310). In the plasma cell neoplasm 70429, azaserine resistance is apparently not caused by loss of the capacity for *de novo* synthesis nor, in extracts of resistant cells, by insensitivity of this pathway to inhibition, although these characteristics may be quantitatively altered (300). There is, however, an "intact cell effect"; with cell suspensions there was a greater difference between the sensitive and resistant lines in degree of inhibition than there was with extracts. Since there was some inhibition in resistant cells, azaserine did enter these cells, and it was emphasized that the results do not necessarily imply a permeability difference. Transport studies have so far indicated very little difference between sensitive and resistant tumor lines in uptake of azaserine or diazo-oxo-norleucine (311, 312); a resistant line of leukemia L1210 actually appeared to take up more of the latter than the sensitive line (312).[11] As originally indicated, the "intact cell effect" in 70429 could be explained by differences between whole cells and extracts in capacity to inactivate the analogue or render it unavailable to sensitive enzyme systems (cf. amethopterin; *vide supra*). Baker (314) recently postulated as an explanation a difference in capacity to produce pyridoxal phosphate as a cofactor for glutamine amide transfer; purified preparations of glutamine-requiring amination enzymes have shown no requirement for pyridoxal phosphate, but other differences of this sort at the cofactor level are possible. Thus, in the case of compounds like amethopterin and azaserine, which can affect enzymatic reactions directly without further anabolism, resistance probably cannot depend upon loss of capacity for analogue anabolism, as it can with purine and pyrimidine analogues, and here the cell may turn instead to other mecha-

[11] These analogues were effectively concentrated intracellularly, apparently by the amino acid active transport system (309, 313).

nisms, such as cell entry or analogue "binding" or inactivation, to protect sensitive enzyme systems.

Other anticancer agents.—Alkylating agents have been discussed in other recent reviews (50, 163, 165, 315). It has been recently reported that mitomycin selectively inhibits DNA synthesis (316) and that there is cross-resistance in sarcoma lines between mitomycin and certain alkylating agents (317).

Jesse Phillip Greenstein

On February 12, 1959, the community of cancer investigators lost one of its great figures with the death of Jesse Phillip Greenstein. For more than two decades he had made important contributions to cancer biochemistry through his own research, his advice to investigators, and his writing and editorial activities. Along with his contributions to biochemistry and nutrition generally, he enriched our knowledge and understanding of biochemical aspects of the cancer process, both in cancer tissues and in tumor-bearing hosts; his book, *Biochemistry of Cancer*, continues to influence cancer research significantly, and his publications included important reviews on cancer enzymology, nucleoproteins, and amino acid resolutions. The field of cancer biochemistry owes much to Dr. Greenstein, and his loss is strongly felt by many investigators, several of whom were privileged to have received from him inspiration, knowledge, and an appreciation for the significance of sustained effort in research.—Carl G. Baker, National Cancer Institute, December, 1959.

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IMMUNOCHEMISTRY[1]

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Immunochemistry provides us with a unique tool by means of which we can interfere, in a systematic manner, with a fundamental biochemical process, the biosynthesis of proteins. Although this process can be inhibited by numerous substances, it cannot be modified in a predictable manner except by the administration of antigens. Landsteiner has proved convincingly that the immunological specificity of antigens is, essentially, the chemical specificity of certain determinant groups of the antigen. Hence, the combination of antigens with homologous antibodies must be attributed to a close fit of the combining groups, i.e., to mutual complementariness of the determinant group of the antigen and of the combining group of the antibody. The combination of antigen with antibody is a much simpler reaction than the interaction of an enzyme with its substrate, since antigen-antibody interaction is free from complications by the catalytic action of an enzyme and by the conversion of a substrate into its products.

Although it is easy to induce the formation of antibodies, to isolate some of them, and to investigate the combination of antigen with antibody, many of the fundamental problems of immunochemistry are still unsolved. We do not yet know all prerequisites of antigenicity, nor the nature of the combining groups of the antibodies. Moreover, it is not yet clear whether the antigen, after eliciting antibody formation, is dispensable in later phases of the immunological response. The basic problems of immunochemistry have been reviewed in earlier articles (1 to 7a) and form the background of the following discussion. Problems of allergy and of the role of histamine, serotonine, and other similar agents have been surveyed in another Annual Review (7b); hence, they will not be treated in this review.

Antigens

Immunopolysaccharides.—The determinant portions of the blood-group substances A and B and of many of the bacterial antigens are formed by polysaccharides. Progress in this field of research has been reviewed (8 to 12), so that this section will be limited to the most recent developments in the chemistry of the immunopolysaccharides. As reported earlier (2, 9, 10), the human blood-group substances A and B, contain fucose in addition to glucose, galactose, and glucosamine. Fucose seems also to be present in the blood-group substance Le^a; the action of anti-Le^a sera is inhibited by lactofucopentaose, a pentasaccharide in which the α-L-fucosyl residue is attached

[1] The survey of the literature pertaining to this review was concluded in October, 1959.

by a 1, 2-bond to the terminal galactosyl residue of β-D-galactosyl-(1, 3)-β-D-N-acetylglucosaminoyl-(1, 3)-β-D-galactosyl-(1, 4)-D-glucose [Morgan and Watkins, (12, 13)]. It had been assumed for many years that persons of the blood group AB contain in their erythrocytes the A as well as the B substance. Morgan & Watkins (14) have now demonstrated that most of these erythrocytes contain AB molecules which react with both types of isoagglutinins. The H-active substance in the red blood cells of persons of the blood group O is probably a precursor of the substances A and B, which are formed from H by the action of the A and B genes; in presence of the O, gene H remains unchanged (12). Enzymes which liberate galactose and N-acetylglucosamine from the A and the B substance and convert them into the H substance have been detected in *Trichomonas foetus* (15) and in *Clostridium tertium* (16, 17).

Most of the earlier work on bacterial immunopolysaccharides was concerned with the capsular polysaccharides of pneumococci. Heidelberger *et al.* (18) have now demonstrated that immune sera against the pneumococcal types II, IX, XII, XX, and XXII are able to precipitate synthetic polyglucose. Evidently, the determinant group of the polysaccharides of these types of pneumococci contains glucose residues. In the somatic antigens of *Salmonella*, a series of rare sugars has been detected by Westphal (19): paratose (3,6-di-deoxy-D-glucose) has been found in Salmonellae of the group A, abequose (3,6-di-deoxy-D-galactose) in group B, tyvelose (3,6-di-deoxy-D-mannose) in the groups D and E, and colitose (3,6-di-deoxy-L-galactose) in group P. Kabat (20), who detected earlier the antigenic action of dextran in man, has now found that the antibodies formed are heterogeneous; their specificity is directed against 1,2- or 1,3-glucosyl residues in the dextran molecule (21).

Proteins.—Most of the investigations in the area of proteins have been done with native serum albumin and its substitution products. The carboxyl groups of bovine serum albumin (BSA) do not seem to be essential for its antigenicity since methylated BSA combines with anti-BSA; however, the COOH groups are necessary for the precipitation of the antigen-antibody complex [Ram & Maurer (22a)]. Acetylation of rabbit serum albumin makes this protein antigenic for rabbits (22b). If human serum albumin (HuSA) is injected to rabbits, some of the antibodies combine only with products of the partial hydrolysis of HuSA, but not with native HuSA; partial hydrolysis in these experiments has been accomplished by cathepsin A from rabbit spleen [Lapresle *et al.* (23)] or by chymotrypsin [Porter (24)]. We have to conclude from these experiments that not all of the determinant groups of a globular protein antigen need to be present in the surface of the molecule. Gelatin, which for a long time was considered as non-antigenic, shares with dextran (20) and polyvinylpyrrolidone [Maurer (25a)] the property of being antigenic for man (25b). The antigenicity of these substances seems to increase with their molecular weight.

Other antigens.—The synthetic polyamino acids are serologically active

as haptens but cannot induce the formation of antibodies. They can be converted into antigens by coupling with gelatin [Sela *et al.* (26, 27)] or with other proteins [Stahmann *et al.* (28)]. Synthetic poly-D-glutamic acid is identical with the natural polyglutamic acid from bacteria of the anthrax and subtilis group, whereas the analogous poly-L-glutamic acid is serologically inactive [Bruckner *et al.* (29)]. It is surprising that also steroids and other normal products of the animal body act as haptens. If the O-carboxymethyloximes or the succinyl derivatives of estrone or progesterone are coupled to carrier proteins and injected into rabbits, antibodies against these hormones are formed [Erlanger *et al.* (30)]. Some of the haptens which *in vitro* combine with protein are *in vivo* converted into antigens and can then induce antibody formation. The halides and sulfonates of 2,4-dinitrophenol are haptens of this type. Replacement of the acyl residue by H, OH, CH_3, or NH_2 results in the formation of substances which do not combine with proteins; only the two acyl derivatives act as "proantigens" and cause skin sensitivity [Eisen *et al.* (31)].

In view of the great biological importance of the nucleic acids, numerous attempts have been made to produce antibodies against DNA. Since nucleic acids, because of their acidity, combine non-specifically with protamines, histones, and other basic proteins, particular precautions must be taken to avoid false deductions where non-specific complexes may be formed. Recent experiments seem to indicate the formation of antibodies against DNA from the thymus gland [Blix *et al.* (32)] and from *Brucella abortus* [Phillips *et al.* (33)]. It has also been claimed that the blood of patients suffering from lupus erythematosus contains antibodies against DNA [Deicher *et al.* (34)]. Since histones seem to be essential in this reaction [Holborow (35)], non-specific factors may be involved.

Adjuvants.—Freund (36) discovered that antibody formation was considerably enhanced and sustained over longer periods of time when a mixture of the antigen with paraffin oil and killed mycobacteria was injected. Similar effects can be brought about by adsorption of the antigen on alumina, carbon suspensions (37), and quartz particles (38) and also by the injection of the antigen in the form of antigen-antibody aggregates [Adler (39)]. The adjuvants cause local granulomas and inflammatory changes in the regional lymph nodes and in the spleen [Fischel *et al.* (40)], thereby stimulating the formation of antibody-producing cells and increasing the rate of antibody formation.

Labeling of antigens.—If protein antigens are lightly labeled by traces of radioactive isotopes, the serological properties are not noticeably affected. Serum albumin or globulin containing approximately one atom of I^{131} per molecule are eliminated from the circulation at the same rate as the native serum proteins [Knox & Endicott (41); McFarlane (42); Melcher & Masouredis (43)]. Since I^{131} has a half life of only a few days, S^{35} or C^{14} are preferable in long term experiments. S^{35}-sulfanilic acid can be diazotized and coupled to the tyrosyl and histidyl residues of proteins [Ingraham (44); Hauro-

witz & Walter (45)]. S^{35}-dichloroethylsulfone is bound to the amino groups of proteins [Francis *et al.* (46)]. C^{14}-labeled proteins can be prepared by coupling with diazotized 1-C^{14}-anthranilic acid [Crampton *et al.* (47)]. Traces of the same radioactive groups can also be introduced into heavily substituted iodoproteins and azoproteins which contain 20 or more non-radioactive iodinated or azo groups per molecule. Unfortunately, the designation I^{131}-proteins is used for both the trace-labeled and the heavily substituted proteins; they will be designated in this review as I^{131}-proteins (e.g., I^{131}-BSA) and I^{131}-iodoproteins (e.g., I^{131}-iodo-BSA), respectively. Analogous designations will be used to differentiate trace-labeled S^{35}-proteins from the heavily substituted S^{35}-sulfanilazoproteins. Electrophoresis of I^{131}-labeled BSA [Talmage *et al.* (48)] reveals only one peak and a high degree of homogeneity. The reviewer had the same experience with various preparations of S^{35}-sulfanilazo-BSA; evidently most of the protein molecules react to the same extent with the substituting agent.

Both the trace-labeled and the heavily substituted proteins described in the preceding paragraph are externally labeled in contrast to the biosynthetically or internally labeled proteins. These are formed *in vivo* by the incorporation of isotopically labeled amino acids. Immunochemical experiments with internally labeled proteins are complicated by reutilization of the labeled amino acids. When rats are injected with doubly labeled heterologous proteins containing S^{35}-amino acids and traces of I^{131}, their tissue proteins incorporate considerable amounts of the S^{35}-amino acids but contain only traces of I^{131} (49, 50). After nine days, the ratio S^{35}:I^{131} in the liver proteins is about 50 to 100 times higher than in the injected, doubly labeled protein. The bulk of the iodine is excreted through the urine where it is present in the form of iodide, iodinated amino acids, and peptides [Laws (51)]. The S^{35}- and C^{14}-amino acids that are released by the breakdown of the injected, internally labeled antigen are chiefly utilized for the formation of tissue proteins; only a small percentage of the injected S^{35} or C^{14} is reincorporated into the plasma proteins since they constitute only 2 per cent of the total body protein.

Metabolic fate of the antigen.—Radiochemical methods reveal only the presence of the label but not necessarily that of the unaltered antigen. In the reviewer's laboratory, it has been found, however, that S^{35}-sulfanilazo-BGG, (bovine gamma-globulin), one month after injection into rabbits, is present in the hydrolysate of the tissue proteins as a substance which, on chromatography, behaves like an aromatic sulfonic acid (45). Similarly, protein-bound C^{14} in the organs of animals injected with C^{14}-anthranil-azoprotein seems to be present as a derivative of anthranilic acid, since treatment of the protein hydrolysate with ninhydrin yields only small amounts of volatile C^{14}-substances (50).

Heavily substituted iodo- or azoprotein antigens disappear rapidly from the circulation and are deposited at first in the organs of the reticuloendothelial system. Differential centrifugation of liver and spleen homogenates reveals that the protein-bound radioactivity appears within a few minutes in

the microsomal and later in the mitochondrial fraction [Crampton *et al.* (47); Haurowitz & Crampton (52)]. The radioactivity of the nuclear fraction is much lower than that of the cytoplasmic fractions. Similar results are obtained when tissue sections are investigated by autoradiography (53) or when fluorescent (54, 55) or colored antigens (56) are injected. Most of the antigen is found in the cytoplasm of macrophages and lymphoid cells, in plasmablasts, and in reticular cells of the bone marrow. If the cells are damaged by freezing and thawing or by sectioning, the antigen is redistributed; considerable amounts may then be found in the nuclei of the cells (54).

In contrast to the heavily substituted iodo- and azoproteins, the trace-labeled plasma protein antigens circulate a long time in the blood. Three phases in the metabolism of these antigens can be observed: (*a*) circulation, (*b*) clearance, and (*c*) deposition [Crampton *et al.* (57)]. During the initial phase of circulation, equilibration takes place between the intravenously injected antigen and the extravascular body fluids; therefore, the concentration of the antigen in the blood plasma decreases rapidly during the first few days, and equilibrium is established after 65 to 140 hr. [Cohen *et al.* (58)]. Clearance of the antigen from the circulation begins at the onset of antibody formation, i.e., about 5 to 7 days after the intravenous injection of the antigen. Evidently, the newly formed antibody combines with the intravascular antigen to form antigen-antibody complexes which are trapped in the cells of the reticuloendothelial system and cause there an increase in radioactive material (57). If the liver or spleen of the injected animals is homogenized during the initial phase of circulation, the extravascular fluids, which contain high concentrations of the antigen, contaminate the subcellular fractions of the homogenate; this leads to almost equal distribution of protein-bound radioactivity in the subcellular fractions.

If antigens or haptens, labeled by traces of isotopes, are injected into animals which have previously been sensitized against the same antigen, they are eliminated from the circulation much more rapidly and are also metabolized at a faster rate than in normal animals (59 to 62). The rapid disappearance of antigens and haptens in sensitized animals is attributed to combination of the injected material with circulating or intracellular antibody.

Persistence of the antigen.—It would be of great importance to know whether the antigen molecule itself or serologically active fragments persist in the sensitized organism over the entire period of antibody formation or whether antibody production can later take place in the absence of antigens. It has been known for several years that pneumococcal polysaccharides persist in mice for periods of at least one year [Stark (63)] and probably for much longer. This was attributed to the lack of enzymes which could hydrolyze these polysaccharides. Analogous persistence of proteins was considered improbable since most tissues contain proteolytic enzymes; however, injection of azoproteins labeled by traces of C^{14}-anthranilic or S^{35}-sulfanilic acid reveals persistence of the protein-bound label in the parenchymatous organs over periods of several months [Ingraham (44); Crampton *et al.* (47, 57);

Garvey & Campbell (64)]. Persistence of unlabeled bovine serum albumin in the liver of rabbits over periods of two months has also been proved by serological methods [McMaster *et al.* (65)]. Since the injected antigens are soluble, whereas most of the tissue-bound radioactive proteins are insoluble, the tissue-bound material cannot be the original antigen; it is either a denatured, insoluble form of the antigen or an insoluble complex formed by the combination of the antigen with other proteins, polysaccharides, nucleic acids, or lipids. Many of the native proteins are quite resistant to the action of proteolytic enzymes. Their denaturation may be a prerequisite for their proteolytic breakdown (66). Garvey & Campbell (64b) assume that fragmentation of the antigen precedes its combination with RNA. In agreement with this view, we found that some of the S^{35}-BSA injected into rats can be extracted from the spleen homogenates by isotonic citrate solution, which solubilizes ribonucleic acid (67).

The amount of radioactive material found in the tissues of animals sensitized with isotopically labeled antigens is very small; several months after injection of the antigen, the radioactive material drops to an average of several hundred antigen molecules per liver or spleen cell (47, 57). Since not all cells contain the antigen, the amount of antigen per antigen-binding cell may be higher. Obviously, presence of antigen molecules or antigen fragments in a cell does not yet prove that presence of the antigen is necessary for antibody formation. Pigments used for tattooing persist in the cells of the skin for many years; yet they do not act as antigens and do not induce antibody formation. Small amounts of I^{131}-iodide [Masouredis *et al.* (68)] or of the S^{35}-sulfanilazohaptens [Haurowitz *et al.* (69)] persist in the tissue proteins without inducing antibody formation. Neither in these instances nor after the injection of isotopically labeled antigens is the rate of antibody formation related to the amount of persisting label [Friedberg (70)]. Many of the tissue-bound antigen molecules may be non-specifically bound; obviously only those antigen molecules which penetrate into cells involved in the production of antibodies are efficient in antibody formation. At present we are not yet able to differentiate non-specifically bound molecules or fragments of the antigen from those molecules or fragments which, because of their specific location, are able to induce antibody formation.

Antibodies

Detection of precipitating antibodies.—During the last decade, the precipitin reaction has been refined considerably by the application of gel diffusion techniques. In a method first described by Oudin (71), the immune serum is mixed with a warm agar solution, placed in a small test tube or between parallel glass plates, and cooled to form a gel; the antigen solution is then layered over the antibody gel. Diffusion of the antigen into the antibody gel causes the formation of a narrow precipitation band. A great advantage of all gel diffusion methods is the stability of the precipitation band and its resistance to small changes in temperature or to slight motion. Another advantage is the slow development of a continuous concentration gradient of

antigen and antibody which prevents disappearance of the precipitate by dissolution in an excess of antigen. Two or more precipitation bands are formed when two or more antigen-antibody systems are present. The sensitivity of the Oudin method is increased by the "double diffusion" technique in which the agar gel is placed between the antigen and the antibody solution [Oakley & Fulthorpe (72); Preer (73)]. If microtubes with a diameter of 1.7 mm. are used, less than 1.0 μg. of antibody can be detected.

In the Ouchterlony technique, the Oudin tubes are replaced by agar gels in flat plates (74). Solutions of the antigen and antibody are placed in small bottomless glass cups or into holes in the agar gel. The method allows investigation of the simultaneous diffusion of one or more different antigens into the examined immune serum. Unrelated antigen-antibody systems give independent precipitin bands, whereas the precipitin bands of cross-reacting systems fuse and form branches [Wilson & Pringle (75)].

The presence of antibodies can also be detected by paper or agar gel electrophoresis of the immune serum in the presence of antigen. The soluble antibody molecules migrate in the electrical field according to their electrical charge, whereas the insoluble antigen-antibody complex does not move, thereby revealing the presence of antibodies against the added antigen [Buttery (76)]. The designation "immuno-electrophoresis" has been coined for a combination of horizontal gel electrophoresis with subsequent diffusion of the immune serum from either side of the antigen-containing strip of the gel. Grabar (77a) and Grabar & Williams (77b) detected, by means of this method, a multiplicity of antigens in normal and pathological blood sera and a corresponding number of antibodies in immune sera.

Isolation and purification of antibodies.—Since the antibodies are gamma-globulins, they can be separated from other serum proteins by all those methods which are suitable for the preparation of gamma-globulins. Further fractionation and purification can be accomplished by partition chromatography with potassium phosphate and cellosolve at −3° [Humphrey & Porter (78)]. If a single type of antibody is to be separated from other antibodies, specific methods of isolation must be used. Most of these methods make use of a resin to which antigen or haptenic groups identical with those of the antigen have been firmly bound. If an antibody solution is passed through a column of this type, the antibody molecules are specifically adsorbed. Subsequent elution yields a pure solution of the antibody. Serum-albumin columns have been prepared by treating carboxyl resins with thionylchloride and by combining the protein with the COCl groups of the chlorinated resin [Isliker (79)]. In another procedure, serum albumin is bound to diazotized aminobenzylcellulose [Campbell *et al.* (80)]. Both preparations adsorb specifically antibodies to serum albumin; the purified antibody is eluted at pH 3.2. The columns of cellulose powder can also be replaced by filter paper which is directly nitroalkylated with *m*-nitrobenzyl-oxymethylpyridinium chloride

$$(NO_2 \cdot C_6H_4 \cdot CH_2 \cdot O \cdot CH_2 \cdot NC_5H_5)^+Cl^-,$$

then reduced with hydrosulfite, diazotized, and coupled to the antigen; antibodies are specifically bound by such an "immunosorbent" [Gurvich *et al.* (81)]. Antibodies to synthetic haptens can be adsorbed to cellulose preparations which are substituted by the homologous hapten group; the adsorbed antibodies are then eluted by a solution of the free hapten, and the latter is removed by dialysis [Karush & Marks (82)]. If the antigen does not lose its specificity by combination with homocysteine thiolactone, the antigen-antibody precipitate can be isolated, dissociated by lowering pH to 2.4, and the antigen then precipitated by 3,6-*bis*(acetomercuromethyl)dioxane; the purified antibody remains in solution [Singer *et al.* (83)]. Antibodies against specific tissue antigens can be purified *in vivo;* if anti-kidney antibodies are injected into rats, they are preferentially adsorbed to the kidneys and can then be split off from the kidneys by perfusion with dilute alkali [Pressman & Sherman (84)].

As reported in a previous review (9), purified antibodies have the same, or a very similar, amino acid composition as normal gamma-globulins and also the same N-terminal amino acid sequence. In the reviewer's laboratory, no significant difference was found between the amino acid composition of antibodies to azoproteins containing either acidic or basic determinant groups (85). Although a crystalline diphtheria antitoxin had been described earlier, preparations with higher antitoxin titer are amorphous [Pope & Stevens (86)]. Recently, Porter (87) has shown that antibody after exposure to the action of papain is split into three fragments which can be separated by chromatography on carboxymethylcellulose. One of these three subunits has been obtained in a crystalline form; it has also been found in normal gamma-globulins and has no affinity to the antigen. The two other peptide fragments combine specifically with the antigen (87). If antibody to an azoprotein containing the *p*-azobenzoate residue is treated with papain, according to Porter, each of the two subunits combines with one hapten molecule, whereas the original antibody molecule combines with two hapten molecules [Nisonoff & Woernley (88)]. Evidently, each of the subunits has a single specifically combining anti-azobenzoate group.

Labeling of antibodies.—Isotopically labeled antibodies have been used for the detection of small amounts of antigen in solutions and for the localization of antigens and antibodies in tissue sections. Labeling has been accomplished by iodination with I^{131} and by coupling with diazotized S^{35}-sulfanilic acid or with S^{35}-dichlorodiethylsulfone, i.e., by methods which have been used for the labeling of antigens (see the first section of this review). The combining group of the antibody can be protected by addition of an excess of hapten [Pressman & Sternberger (89)]. Tritiated antibodies have been produced by injection of α,β-tritiated valine to sensitized animals [Crawhall *et al.* (90)]. Very small amounts of the labeled, radioactive antibodies can be detected by adding non-radioactive antibody as carrier and precipitation with the homologous antigen.

Although isotopically labeled antibodies are valuable for the investiga-

tion of antigen-antibody interaction in solution, labeling of antibodies by fluorescent substituents [Coons (6); Coons *et al.* (55)] has gained much more importance in the localization of antigens and antibodies in tissue sections. The great advantage of this method is that the fluorescent material can be seen in the fluorescence microscope and that much less time is required for this method than for autoradiography. Fluorescein isocyanate, used originally by Coons for labeling antigens or antibodies, is at present frequently replaced by the more stable fluorescein isothiocyanate [Marshall *et al.* (91)]. Fluorescent antibody was originally a tool for the detection of antigen in tissues; if it is used for the detection of antibody, the tissue section is first frozen, treated with ethanol to make antibody less soluble, and then covered with an excess of the antigen so that antigen-antibody complexes are formed; after removal of the excess of free antigen, the section is exposed to the fluorescent antibody which combines with the bound antigen and thereby reveals the localization of antibody in the tissues. More than one antigen or antibody can be localized by using antibodies labeled with different fluorescent substituents. Whereas fluorescein displays a bright green fluorescence antibodies coupled with rhodamine derivatives show an orange fluorescence (92). Fluorescent antibodies have also been prepared by coupling with 5-dimethylamino-1-naphthalene (93). The fluorescent antibody method can be simplified considerably by first using non-fluorescent human (or rabbit) antibodies against the applied antigen, and then applying fluorescent antibody of another animal species against human (or rabbit) serum gamma-globulin; a single fluorescent type of antibody is then sufficient for all types of antigen-antibody systems [Carter & Leise (94)]. Localization of antibodies in electronmicrographs can be accomplished with ferritin, a protein which contains more than 20 per cent iron; it is coupled to the antibody protein by means of *m*-xylylene-diisocyanate, $C_6H_4(CH_2NCO)_2$ [Singer (95)].

Non-precipitating antibodies.—Non-precipitating antibodies have been neglected in immunochemical research because it is difficult to determine them quantitatively. Yet their clinical importance is at least as great as that of the precipitins. The non-precipitating antibodies responsible for allergic phenomena are frequently called reagins. Blocking antibodies, found in about 50 per cent of the Rh-negative mothers of erythroblastotic infants, compete with precipitins and agglutinins and thereby prevent visible antigen-antibody interaction. According to Kaplan & Dienes (96), some of the non-precipitating antibodies, particularly those responsible for the delayed type of hypersensitivity, may be products of the first phase of antibody formation which is later followed by the formation of precipitating antibodies (see also 97, 98). The immunological importance of the non-precipitating antibodies has been discussed in several reviews (99, 100). Only the biochemical aspects will be considered here. Kuhns & Pappenheimer (101) discovered three types of antibodies in the serum of persons injected with diphtheria toxoid: (*a*) precipitating, (*b*) skin sensitizing, and (*c*) non-sensitizing. The two latter types were non-precipitating; only the non-sensitiz-

ing antibodies were blocking. The skin-sensitizing antibodies were found in the gamma$_1$-globulin fraction and the other two types, in the gamma$_2$-globulins (101). The blocking antibodies in the sera of allergic persons migrate in electrophoresis with the gamma-globulins whereas the reagins migrate with the beta-globulins [Cann & Loveless (102)].

The amount of non-precipitating antibody can be estimated from its neutralizing action if the homologous antigen is an enzyme, hormone, or toxin or if it has another biologically measurable activity which decreases on combination with the antibody. The non-precipitating antibodies are frequently called univalent or incomplete antibodies. The former designation is objectionable since the "valence" of antibodies is only one of several factors which determine whether visible precipitation or agglutination occurs. Thus, non-agglutinating antibodies agglutinate the homologous red blood cells after exposure of the cells to trypsin (103, 104) or papain (105). Visible agglutination by incomplete antibodies is brought about in Coombs' test (106), in which the red blood cells are first coated with human blocking antibodies and are then agglutinated by rabbit anti-human antibodies. Similarly, visible agglutination results when incomplete antibodies act on red blood cells to which the antigen has been attached by means of tannic acid [Boyden (107)], by *bis*-diazotized benzidine [Pressman *et al.* (108)], or by formaldehyde [Ingraham (109)]. One of the most sensitive methods for the detection of the non-precipitating antibodies is the PCA (passive cutaneous anaphylaxy) test in which guinea pigs receive an intracutaneous injection of antibody and six hours later an intravenous injection of antigen and Evans Blue; if the intradermally injected solution contains antibody, the skin around the site of injection is stained blue [Ovary (110)]. The PCA test is about 1000 times more sensitive than the customary quantitative precipitin methods; as little as 0.02 μg. of antiovalbumin can be detected by the test (110).

The site of antibody formation.—Earlier objections of the reviewer (111) against claims that antibody can be manufactured *in vitro* have now been confirmed (112). It seems that only cells of vertebrates are able to produce antibodies. Attempts to produce antibodies in invertebrates have failed (113). Frogs form antibodies but do not release them into the circulation at temperatures lower than 8° C. [Bisset (114)]. In the mammalian organism, antibodies can be formed in various organs, depending on the site of administration of the antigen [Askonas *et al.* (115)]. After intravenous injection, antibody formation begins in the spleen and is later taken over by other organs of the reticuloendothelial system [Taliaferro (116)]. Subcutaneous or another form of local administration leads to the formation of antibodies at the site of injection and in the regional lymph nodes [Thomson *et al.* (117)]. Since antibodies are gamma-globulins, persons suffering from agammaglobulinemia cannot form antibodies. Using his fluorescent antibody technique, Coons *et al.* (55), in agreement with earlier work of Fagraeus (118), found the highest concentrations of antibody in plasma cells which, because of their

high RNA content, have an affinity for pyronine and other basic dyes. The presence of antibody in the plasma cells has also been confirmed by autoradiography with isotopically labeled antigen [Berenbaum (119)]. The precursors of the plasma cells are cells of the reticular system [Fitch *et al.* (120)], probably lymphoid cells [Roberts & Dixon (121)]. Transfer experiments with lymph node cells of sensitized animals reveal that 150 to 350×10^6 antibody molecules are produced per viable lymphocyte, i.e., approximately one-third of the weight of the transferred cells (121).

Auto- and isoantibodies.—The definition of antibodies involves antigens as causative agents. This definition fails when applied to isoantibodies, for instance human isoagglutinins; their formation seems to be directed by genetic factors without any interference by true antigens. Autoantibodies react with proteins of the same organism. Autoantibodies against the lens of the eye have been known for many years. Similarly, proteins of the thyroid gland, if injected with an adjuvant, cause the formation of autoantibodies [Witebsky & Rose (122)]; autoantibodies of this type have been detected in Hashimoto's disease [Roitt *et al.* (123)]. Grabar (124) assumes that massive degradation of body cells results in modifications of the normal proteins which thereby are converted into antigens and induce the formation of autoantibodies.

Kinetics of antibody metabolism.—The half life of antibodies can be determined by injecting isotopically labeled antibodies into normal animals and measuring their elimination from the blood plasma. Heidelberger and Schoenheimer (1942) had used N^{15}-labeled antibodies for this purpose. Humphrey & McFarlane (125) prepared C^{14}-labeled antibodies by administering C^{14}-phenylalanine to rabbits injected with pneumococcal polysaccharides. The immune sera of these animals were injected intravenously into normal rabbits. Although the antibody content decreased in the recipient animals corresponding to a half life of about six days, the specific activity (cts. per min. per mg.) of the antibody remained unchanged. This proves that the circulating antibody molecules do not exchange their C^{14}-phenylalanine residues. Similar half lives of rabbit antibodies were observed in animals injected with C^{14}-valine [Gros *et al.* (126)] and in antibodies labeled externally by traces of I^{131} [Tekman & Dalgliesh (127)]. Half lives of I^{131}-antibodies in different species are: mouse 2 days, guinea pig 5, rabbit 5, dog 8, man 13, and cattle 21 days [Dixon *et al.* (128)].

If tissue slices of sensitized rabbits are incubated with labeled amino acids, rapid incorporation into antibody and other proteins is observed; however, the newly formed antibody remains 10 to 30 min. inside the cells and appears only after this period of time in the serum [Askonas & Humphrey (129)]. Antibody is formed only in intact cells, not in homogenates. The rate of antibody formation in the rabbit spleen is approximately 32 μg. of antibody per gram of spleen in 3 hr. (129). Within the cells, the antibody is found in the microsomes and can be released by disruption of the cells (129). The total amount of antibody formed exceeds by far the amounts of antigen

required for sensitization; after injection of 0.36 μg. of diphtheria toxoid, at least 10^6 molecules of circulating antibody per molecule of injected toxoid were formed in a period of two weeks [Cohn & Pappenheimer (130)]. However, the capacity of the organism to form antibodies is not unlimited; if two different antigens are injected simultaneously, the total amount of antibody formed is less than the sum of antibodies formed in animals injected with one or the other of the two antigens [Abramoff & Wolfe (131)].

In animals injected with a single dose of a soluble protein antigen, such as bovine serum albumin or ovalbumin, the antibody titer reaches a peak 10 to 12 days after injection and then decreases slowly. Three to four weeks after the first injection of antigen the precipitin test is again negative; if at that time or later a small dose of antigen is reinjected, much larger amounts of antibody are formed than in the primary response. The antibody titer, which rises to a maximum five to seven days after injection, decreases very slowly; even many months after the second injection, antibody can be detected [Dixon *et al.* (132)]. This "secondary response," also designated as "anamnestic reaction," is highly specific and can be elicited only by an antigen that is identical with, or closely related to, the antigen that caused the primary response. If the antigen used for the secondary response is not identical but only related to the first antigen, its injection may lead to the reappearance of antibodies against the first antigen [Dubert (133)].

Measurements of the specific activity of antibody of the primary and secondary response in animals injected with labeled amino acids suggest that some of the rapid increase in the antibody titer of the secondary response may be caused by the release of preformed antibody from the tissues into the circulation [Cheng & Haurowitz (134); Patras (135); Garvey & Campbell (136)]. However, the amount of antibody released in this manner cannot be very large since the specific activity of S^{35}-antiovalbumin formed in a secondary response to OA is almost the same as that of S^{35}-antibovine serum albumin formed in a simultaneous primary response to bovine serum albumin [Richter & Haurowitz (137)]. The formation of antibody at least one to two days prior to its appearance in the blood serum is also indicated by experiments in which two or three different isotopic labels were injected before, or shortly after, the secondary stimulation [Patras (135); Green & Anker (138)]. The interpretation of the experiments with isotopically labeled amino acids is complicated by the reutilization of these amino acids after the breakdown of labeled tissue proteins. Gurvich (139) and Taliaferro & Taliaferro (140) conclude from their results that all of the antibody found in the anamnestic reaction is formed in response to the reinjection of antigen and that there is no significant release of preformed antibody. Presence of small amounts of antibody before the secondary stimulation is, however, indicated by the continual formation of antibody observed in other experiments. Heidelberger *et al.* (141) found antibody to pneumococcal polysaccharides many years after the immunization of volunteers. Similarly, antibodies to ovalbumin or bovine serum albumin can be detected in the serum of rabbits many months

after a single primary injection, provided the sensitive hemagglutination test is used instead of the customary precipitin reaction (142).

It is not yet known if this continual formation of antibodies is to be attributed to the persistence of antigen. It is, however, clear that the secondary injection of an antigen must lead to the immediate formation of antigen-antibody complexes. The injected antigen seems to combine not only with circulating antibody molecules but also with tissue-bound antibody [Francis & Hawkins (143)]. Najjar & Fisher (144) have shown that antibodies formed in response to antigen-antibody complexes differ slightly from antibodies formed in response to soluble antigens. The antigen-antibody complex seems to act like an antigen-adjuvant complex and is a more powerful stimulant of antibody formation. The reviewer attributes the intensity and rapidity of the secondary response to the rapid combination of the injected antigen with preformed antibody.

Cultivation and transfer of antibody-forming cells.—It has been known for many years that the tissues of sensitized animals continue to produce antibodies if incubated *in vitro* under suitable conditions [Kimura (145)]. If isotopically labeled amino acids are added to such a culture of spleen or of lymph nodes, the small amount of newly formed antibody can be measured quantitatively. Steiner & Anker (146) obtained high yields of antibody by cultivating the cells on a dialysis membrane that separated them from the nutrient solution. Damage by rolling or shaking was avoided in this manner. Lymph node cells are about twice as active as spleen cells; liver cells have about one-fifteenth of the activity of those from the spleen [Keston & Katchen (147)]. In evaluating this figure, it must be kept in mind that the weight of the rabbit liver is about 20 to 50 times higher than that of the spleen.

If the spleen or lymph node cells of a sensitized rabbit are injected into a normal rabbit, antibody formation is observed in the recipient, even if the antibody-forming capacity of the recipient is suppressed by prior exposure to x-rays. Evidently, the antibodies are formed in the transferred cells of the donor and not in the cells of the irradiated recipient [Harris *et al.* (148)]. The irradiated animals are not able to form antibodies against the foreign cells which persist for many weeks (148); this can be considered as colonization in the irradiated organism, i.e., as "radiation chimera" [Mitchison (149)]. It is a prerequisite for antibody formation by the transferred cells that the cells are not removed from the sensitized donor earlier than 10 min. after injection of the antigen (148). Apparently, a certain minimum time is necessary for the penetration of the antigen into cells which are involved in the production of antibodies. Viable Shigellae, which had been used in earlier experiments as antigens, were later replaced by a filtrate of trypsin-treated Shigellae (148) or by heterologous plasma proteins [Dixon *et al.* (150)]. Injection of S^{35}-amino acids to the recipient or to the donor animals demonstrates convincingly that the bulk of the antibodies is formed from amino acids of the recipient [Taliaferro & Talmage (151)]. Agglutinin formation has been ob-

served in irradiated rabbits into which a mixture of normal rabbit cells and *Shigella* antigen was injected [Harris & Harris (152)] and also in newborn rabbits injected with adult rabbit spleen cells that had been incubated *in vitro* with *Salmonella paratyphi* [Šterzl (153a)]. In both instances, antibody production is attributed to the injected cells. Although the amount of antigen bound by the adult cells during incubation is very small, it seems to be essential for antibody formation; the transferred cells stop forming antibody when the antigen is used up; they resume antibody production after the injection of more antigen [Šterzl (153b)]. Stevens & McKenna (154) have recently reported that the diced spleen of normal rabbits forms antibovine gamma-globulin *in vitro* after incubation for one hour with bovine gamma-globulin provided the rabbits, before removal of the spleen, were given an intravenous injection of 10 μg. of the endotoxin of *Salmonella typhosa*. Confirmation of these results would be desirable, since earlier attempts to produce antibodies by adding antigen to tissue cultures of lymph node or spleen cells had no success.

Since irradiated animals cannot form antibodies, colonization of the injected cells takes place even if they are of heterologous origin. Thus, bone marrow from rats survives after transfer to irradiated mice and leads to the production of rat erythrocytes in the organism of the mouse until the murine bone marrow is regenerated (155, 156). The injection of rat bone marrow into mice is reminiscent of the transplantation of homografts. In adult recipients which were not irradiated, the grafted tissue is destroyed because of the formation of antibodies against antigens in the transplanted material. The nature of these antigens is not yet known though they are probably proteins; the suggestion has been abandoned that the DNA of the transplanted tissue acts as an antigen (157, 158). Since most of the homografts contain also antibody-forming cells, antibodies against the host may be formed in the homograft in addition to anti-homograft antibodies formed in the cells of the host.

Immunological unresponsiveness.—Felton (159) observed, in 1949, that mice injected with more than 0.5 mg. pneumococcal polysaccharide of the type III lost their immunity against pneumococci for periods of more than one year. The phenomenon has been designated as "immunological paralysis." It is caused by the resistance of the antigen to enzymatic action. Consequently, persistence of antigen in the tissues may result in a permanent excess at the antibody-forming sites and immediate neutralization and breakdown of the newly formed antibodies [Dixon *et al.* (160)]. Similar "tolerance" can be produced in rabbits by injection of very large doses of bovine or human serum albumin [Dixon & Maurer (161)]. The immunological paralysis or tolerance is highly specific; formation of antibodies against other types of antigen is not affected. The duration of the unresponsiveness increases with the amount of injected antigen [Smith & Bridges (162)]; it probably lasts as long as the retained antigen is sufficient to neutralize all of the antibody formed during a period of two half lives (163).

The inability of newborn animals to produce antibodies makes it possible to produce tolerance of long duration by injecting the animals with very

small amounts of the antigen [Billingham *et al.* (164); Hanan & Oyama (165)]. Immunological unresponsiveness in rabbits lasts about eight to ten days after birth [Dubert & Paraf (166)]. From that time on, the animals react like adult animals and form antibody after the injection of antigen. The unresponsiveness of the newborn animals is not caused by their inability to retain the antigen; I^{131}-proteins are deposited in the tissues of newborn rabbits to the same extent as in adult rabbits [Crampton *et al.* (167)]. If the spleen cells of newborn rabbits are incubated with trypsinated *Shigella* antigen and then injected into x-rayed rabbits, antibodies are formed [Dixon & Weigle (168)]. This proves that the spleen cells of the newborn rabbit are able to form antibody in a suitable environment. Indeed, antibody formation is observed in newborn rabbits when bacterial endotoxins are used as antigens [Šterzl & Trnka (169)]. It seems that irritation and cellular damage caused by the bacterial toxins enhance the maturation of antibody-forming cells or interfere with the action of an unknown inhibitor. Finally it may be mentioned that unresponsiveness can be produced in adult animals not only by irradiation with x-rays but also by the administration of 6-mercaptopurine [Schwarz & Dameschek (170)].

The mechanism of antibody formation.—The production of modified proteins (antibodies) that are complementarily adjusted to the injected antigen is one of the most interesting problems of biochemistry. At a time when only bacterial and other natural antigens were known, Ehrlich (1906) proposed that small amounts of antibodies for all possible antigens are preformed in the organism and that injection of an antigen stimulates accelerated regeneration of the analogous type of antibody. It became difficult to reconcile this view with the subsequent discoveries of Landsteiner, as it would necessitate the assumption that the body has preformed antibodies against the numerous synthetic azoaryl haptens. Breinl and the reviewer (1930) suggested, therefore, that the antigen interferes with the normal process of globulin formation in such a manner that globulins (antibodies) are formed the shape of which is complementarily adjusted to the determinant (haptenic) group of the antigen. Similar views were proposed at the same time by Mudd and by Alexander. In 1940, Pauling accepted these views and suggested that complementariness of the antibody globulin is accomplished by suitable folding of the peptide chains rather than by differences in the number or sequence of the amino acids. This suggestion is supported by recent amino acid analyses and forms an essential part of the "template theory" of antibody formation (171). Askonas & White (172) and Dixon (173) have pointed out that antibody formation takes place in the plasma cells and not in those cells which normally form gamma-globulins. Gurvich & Smirnova (174) have postulated that the antigen interferes not only with the folding of the peptide chains but also with the sequence of the amino acids. These objections do not affect, however, the cardinal postulate of the "template theory" that antigen molecules or their haptenic fragments act as templates and must be present during the formation of new antibody molecules. Since antibody formation, as shown in preceding sections, frequently continues for

many months or years, antigen molecules or fragments must persist for the same long periods of time. Although persistence of many of the isotopic labels of antigens has been demonstrated in the reviewer's laboratory, the available analytical methods do not allow us to prove that the isotope is still part of the unchanged antigen or hapten.

The claim that protein antigens persist *in vivo* for many months or years is considered improbable by many workers who prefer to assume that antibody production continues after elimination of all of the antigen molecules. This assumption raises the serious problem of how to explain formation of structures which are complementary to molecules which previously acted as models but were later eliminated. The first attempt to overcome this difficulty was made by Burnet & Fenner (175) who assumed that antibody formation was a process akin to the formation of adaptive enzymes. In the mean time, it has become known (*a*) that this process does not involve true adaptation but rather accelerated formation of enzymes which normally are present only in traces and (*b*) that the formation of these enzymes stops after elimination of the inducing agent. Although this theory appeals to many immunologists, it is not necessary to comment on it since it has been abandoned by its proponent, Burnet, in favor of Jerne's modification of Ehrlich's historical theory of preformed receptors (tissue-bound antibodies). In agreement with Ehrlich's original view, Jerne (176) assumes that small amounts of antibodies of all types are present in the organism, that the injected antigen combines with these, and that the antibody-antigen complex is carried to, and bound specifically by, those cells that are able to produce antibodies of the homologous type. This "natural selection theory" has been further modified by Burnet (177) who proposes a clonal selection theory according to which each antibody-forming cell has the intrinsic capacity to produce only one type of antibody and to transmit this capacity to its daughter cells. The experimental basis for this view is the occurrence of clusters of antibody-forming cells in lymph nodes and spleen in the secondary response. Burnet's theory was tested by Nossal & Lederberg (178, 179) who injected into the foot pad of rabbits two different types of *Salmonella*, *S. typhi* and *S. adelaide*, isolated single cells from the regional lymph node, and examined the cells for the presence of agglutinins; in 33 out of 228 cells, they found agglutination of *S. adelaide* and in 29, agglutination of *S. typhi*. None of the cells agglutinated both antigens. The same results were obtained by White (180) who sensitized rabbits with ovalbumin and *Streptococcus pneumoniae* and investigated the cells with two different fluorescent antibodies. Contradictory results were, however, obtained by Lennox (181) and Attardi *et al.* (182) who injected rabbits with T_2 and T_5 bacteriophages; they found among 232 cells 15 which formed both anti-T_2 and anti-T_5 antibodies. Hence, the question whether an antibody-forming cell can form more than one type of antibody remains unsettled.

The clonal selection theory of antibody formation is confronted with the same difficulty as Ehrlich's original theory: namely, the necessity to postu-

late preformed antibodies against azophenylarsonate, azophenyltrimethylammonium groups, and other strange artifacts of the organic chemist. This difficulty is not unsurmountable. We know that immunological specificity is not absolute; the complementary adaptation in shape of the antibody can never be perfect. Indeed, most of the antibodies cross-react with a large number of chemically related haptens. The combining group of the antibody molecule contains probably not more than three or four amino acids. Even if it is assumed that all natural amino acids can occur in this group, not more than about 5×10^4 combinations are possible. Some of these might cross-react with the artificial haptens. The number of different combining groups obviously would increase considerably if different modes of folding were accepted for each of the combining tri- or tetrapeptides. We would then have to postulate 10^6 or more types of antibody-forming cells, each producing only one type of antibody. Since we can easily induce the simultaneous formation of different types of antibodies in an individual, the problem of a sufficient number of antibody-forming cells arises. Attempts have been made to raise several generations of antibody-forming cells by tissue culture and to decide whether the daughter cells form antibody. Such attempts have failed since the cells lose their antibody-forming property on multiplication.

The proponents of the clonal selection theory point out that the template theory cannot explain the intensity of the anamnestic reaction and tolerance in newborn animals. In the preceding sections of this review, attempts have been made to explain these phenomena without postulating the presence of specific clones of antibody-forming cells and without invoking the continual formation of all types of preformed antibodies. The clonal selection theory does not make any statement about the fate of the injected antigen or about the length of its persistence in the antibody-forming cell. Indeed, the persistence of the antigen is irrelevant according to this theory since the cell produces small amounts of antibody even before injection of the antigen. The reviewer as a biochemist, prefers to attribute to the antigen molecule the role of a direct template, to postulate its persistence during the time of antibody formation, and to consider antibody formation merely as gamma-globulin formation in the presence of antigen molecules. Burnet considers his clonal selection theory as an "attempt to avoid the necessity of postulating biochemical processes for which as yet there is no independent evidence." A final decision between the two extreme views is not possible at present.

Antigen-Antibody Interaction

Combination of antibody with haptens.—The combination of antibody with small, univalent haptens is the simplest of all immunochemical reactions since it is not complicated by problems of solubility, takes place in homogeneous solution, is reversible, and can be investigated quantitatively by means of equilibrium dialysis. Earlier experiments of this type had led to erroneous results since some of the azo dye haptens exist in aqueous solutions as polymers. Equilibrium dialysis of purified antibodies against D- or L-

phenyl-)*p*-azobenzylamino)acetate shows that the free energy of combination, ΔF, is -7 kcal. per mole of hapten; the entropy change, ΔS, is close to 0 [Karush (183)]. The α-phenyl group contributes 4 and the benzylamino group, 2.4 kcal. to the ΔF value. Similar experiments with the hapten *p*-(*p*-dimethylaminobenzeneazo)-phenyl-β-lactoside gave a ΔH value of -10 kcal/mole; at a large excess of hapten, 1.8 moles of hapten were bound per mole of antibody (183). Using various substituted azobenzoate and azopyridine carboxylates, Nisonoff & Pressman (184) have found that the carboxyl group contributes -4.8 and the *p*-phenylazo group, -2.3 kcal. to the ΔF value; the contribution of the nitro group in azo-nitrobenzenes is -4.0 to -5.3 kcal. per mole of hapten (185). Antibodies to the 2,4-dinitrophenyl group bind two molecules of the hapten dinitrophenyl-lysine but only one molecule of dinitrophenyl-aniline [Carsten & Eisen (186)]. The affinity of antibody for azohaptens decreases when —S—S— bonds of the antibody molecule are reduced to -SH by mercaptoethylamine [Karush (183)]. The large contribution of the carboxyl group of the hapten to the free energy of combination has led to the view that the combining site of the antibody molecule contains a basic group [Epstein & Singer (187)]. However, acetylation of the antibody does not affect combination with the hapten [Nisonoff & Pressman (188)]. Hence, imidazole groups, rather than amino groups, of the antibody seem to be involved in the combination of antibody with haptens or protein antigens [Marrack (189); Turnbull (190)].

Soluble antigen-antibody complexes.—These are either complexes formed by non-precipitating antibodies or complexes consisting of precipitating antibody and an excess of antigen. Ultracentrifugation of such soluble complexes indicates that the molar ratio of antigen/antibody is always less than 2.0 [Singer & Campbell (191)]. Determination of the equilibrium at different antigen/antibody ratios gives ΔF values of about -5 kcal. per mole of antigen and a positive entropy of combination; ΔH is close to 0 (191). Similar values are obtained with arsanilazo-bovine gamma-globulin as antigen [Baker *et al.* (192)]. Light-scattering data, however, indicate that serum albumin combines not only with the homologous antibody but also with normal gamma-globulins [Winkler *et al.* (193)]. Moreover, calorimetric measurements give for the combination of human serum albumin with rabbit antibody a ΔH value of 3.5 kcal. per mole of antigen or 7 kcal. for the formation of an antibody-antigen complex of composition $Ab.Ag_2$ [Steiner & Kitzinger (194)]. The interpretation of the thermodynamic data is difficult since combination may take place not only by bonds between specific sites of the antigen and antibody molecules but also by nonspecific protein-protein interaction particularly when immune sera are used instead of solutions of the purified antibody. Most of the equations proposed for soluble antigen-antibody systems are based on the assumption that all haptenic groups of an antigen molecule are identical and that also all antibody molecules are identical. We know, however, that antibodies form a heterogeneous population and also that the determinant groups of a multivalent antigen molecule, although of identical structure, are heterogeneous, some of them easily accessible to the

antibody molecules, others buried in folds of the peptide chains. In view of these complications, the reviewer believes attempts to express antigen-antibody interactions by mathematical expressions are premature.

Insoluble antigen-antibody complexes.—Precipitation of an antigen by the homologous antibody is generally attributed to the formation of an antigen-antibody framework consisting of "multivalent" antigen and "bivalent" antibody molecules. There is hardly any doubt that most, if not all, antigen molecules contain more than one determinant group and are, therefore, multivalent. It is more difficult to make a definite statement about the valence of the antibodies, i.e., about the number of combining sites per antibody molecule. If antibodies were always bivalent, one should expect that injection of an antigen with two different types of determinant groups, X and Y, would lead to the formation of antibodies of the types anti-X, anti-Y, and anti-XY. Although several attempts have been made to discover "heteroligating" antibodies, i.e., antibodies of the type anti-XY, they have never been found [Eisen *et al.* (195); Nisonoff *et al.* (196)]. Difficulties arise also when the high antibody:antigen ratio of the precipitates is taken into consideration. The molar ratio Ab.:Ag., in general much higher than one, has in most instances a value of five to ten, and rises sometimes to values of more than 50. Thus the thyroglobulin-antithyroglobulin precipitate contains 50 to 60 antibody molecules per antigen molecule. It is hardly possible to devise a framework of this composition in which the antibodies are bound by two combining sites. It is also difficult to understand why the antigen-antibody precipitate formed by rabbit immune sera is soluble in an excess of antigen but not in one of antibody, particularly when the antigen is also a gamma-globulin and very similar in its physicochemical properties to rabbit serum gamma-globulin. For this reason, the reviewer preferred to assume that antibodies had only one combining group and that the precipitation was not caused by bridges of bivalent antibody molecules but by the non-specific aggregation of antigen-antibody complexes in which each antigen molecule was surrounded by a number of univalent antibody molecules.

This view has lost much of its appeal by the discovery of two univalent subunits in rabbit anti-bovine serum albumin [Porter (87)] and by the results of equilibrium dialysis and ultracentrifugation of soluble antibody-hapten and antibody-antigen complexes described in the preceding sections. Another argument in favor of bivalence of the antibodies is the apparent specificity of the aggregation of mixed antigen-antibody complexes. If a mixture of two antigens is precipitated or agglutinated by a mixture of the homologous two antibodies, separate clusters of each of the two systems are formed but not mixed precipitates or agglutinates of the two systems [Umezawa (197); Tayeau & Marqueville (198)].

The formation of antigen-antibody precipitates, like that of other non-specific precipitates, is affected by changes in the ionic strength, in pH, and in other properties of the solvent. The combination of antigen with antibody frequently is attributed to the neutralization of charges of opposite sign in the combining sites (187, 199). Since neutral carbohydrates are antigenic,

charged groups of the antigen cannot be a prerequisite for precipitation, although they may facilitate it. Rabbit immune sera yield the maximum precipitate in 0.85 to 1.5 per cent saline solutions, whereas chicken immune sera give the highest amount of precipitate in 8 to 10 per cent saline solution; the increase in weight of the precipitate is probably caused by a salting-out effect at the higher ionic strength [Goodman *et al.* (200)]. In contrast to immune precipitates from horse immune sera, which are soluble in an excess of antibody, precipitates produced by rabbit antibody are almost insoluble or require an enormous excess of antibody for dissolution [Nisonoff & Winkler (201)]. Antibodies of the type present in horse sera are designated as "flocculating antibodies"; however flocculating antibodies act as precipitating antibodies in 20 per cent ammonium sulfate solution, in which both antigen and antibody are soluble [Pope (202)]. Similarly, soluble complexes formed by anti-bovine serum albumin in an excess of bovine serum albumin are salted out by 50 per cent saturation with ammonium sulfate [Farr (203)].

Numerous equations have been proposed for correlating the composition of the antigen-antibody precipitates with the antigen and antibody content of the supernatant solution. Most of these equations hold for a limited range of antigen/antibody ratio but fail in the regions of antigen or antibody excess. Some deficiencies have been overcome by Goldberg (204), who derived his equations from those elaborated for the distribution of branched-chain polymers. However, even this improved theory cannot explain the differences between solubility of rabbit and horse serum precipitates [Spiers (205)]. If antigen-antibody precipitates are washed with 1 per cent saline solution, they lose some of their weight; this has been attributed to a limited "solubility" of the precipitate. Investigation with isotopically labeled antigen or antibody or both has revealed, however, that only antibody is removed by the saline solution [Haurowitz *et al.* (206)]. The process is represented by the reaction:

$$AgAb_n \rightleftharpoons AgAb_{n-1} + Ab$$

Quantitative analysis at different temperatures gave for the reverse reaction a ΔF value of -8 to -10 kcal. per mole; ΔS was positive and ΔH close to 0.

The structure of antigen-antibody precipitates is, in general, not resolved by electron microscopy, since antigen and antibody molecules have similar density and size. Using the iron-containing antigen ferritin, Easty & Mercer (207) found that the antibody molecules formed a halo around the much denser ferritin molecules; the distance between adjacent ferritin molecules was 100 A in the absence and 200 to 400 A in the presence of antibody. If a single layer of bivalent antibodies is assumed, the average length of the antibody molecules would have to be 300 A; alternatively, the result might indicate two layers of 150 A diameter.

If the antigen particles are not molecules but bacteria or cells, the term "agglutination" is used for the formation of an insoluble antigen-antibody complex. Although agglutination by immune sera is highly specific, some cross-reactions have been observed. One of the most interesting is the agglu-

tination of red blood cells of the blood group A by extracts of castor beans, lima beans, and other legumes; the agglutinating agents have been called "lectins" [Boyd *et al.* (208)]. The amount of free and bound human isoagglutinin in suspensions of red blood cells has been determined in Wurmser's laboratory: ΔH was -6.5 kcal. per mole of antibody, and ΔS $+8$ entropy units [Filitti-Wurmser *et al.* (209)]. Electron microscopy of agglutinates reveals seams of approximately 1000 A between adjacent erythrocytes [Katchalsky *et al.* (210)], which would indicate much more than one or two layers of antibody molecules between the agglutinated cells; the widely held view that the agglutinated cells are held together by bridges of bivalent antibody molecules is hardly reconcilable with these findings.

Complement and hemolysis.—If red blood cells are treated with small amounts of agglutinin and then exposed to fresh guinea pig serum, they are lysed. The lytic factor of guinea pig serum, called complement, consists of at least four fractions designated as C′1 (midpiece), C′2 (endpiece), C′3, and C′4. The former two are inactivated at 55°, whereas C′3 and C′4 are heat-stable. If the guinea pig serum is dialyzed at pH 5.4 against a phosphate solution of low ionic strength, C′1 and C′3 are found in the insoluble euglobulin fraction and C′2 and C′4 in the supernatant solution which contains albumins and pseudoglobulins. C′3 is inactivated at 37° by zymosan, a yeast polysaccharide; C′4 is inactivated by ammonia and primary amines. Kinetic analyses of Mayer and his co-workers (211) have revealed that the erythrocytes (E), after combination with antibody (A), combine first with C′1, then undergo the following sequence of reactions:

$$\text{EAC′1} \rightarrow \text{EAC′1,4} \rightarrow \text{EAC′1,4,2} \rightarrow \text{E*}$$

where E* is a damaged red cell which loses hemoglobin and is converted into a "ghost." The first phase of complement action is the binding of C′1 which is an enzyme precursor, a proesterase; it is activated by combining with EA and is then able to hydrolyse tosyl arginine methyl ester [Becker (212)]. The presence of this esterase was revealed by the discovery that hemolysis is inhibited by DFP (diisopropyl fluorophosphate) [Levine (213)]. The complex EAC′1 inactivates C′2 and C′4 in the presence of calcium and magnesium ions; ethylenediamine tetraacetate inhibits the reaction and thereby prevents hemolysis [Mayer & Levine (214)]. Very little is known about C′3. Rapp *et al.* (215) were able to fractionate complement by means of DEAE-cellulose; instead of C′3, they found two substances, designated as C′a and C′b, which may be components of C′3.

The action of complement is far from being understood. It is particularly obscure why complement causes the lysis not only of sensitized red blood cells but also of red cells which are coated by arsanil- or sulfanilazoprotein and then exposed to anti-azoprotein [Ingraham (216); Silverstein & Maltaner (217)]. Complement combines not only with sensitized cells but also with antigen-antibody precipitates. The solubility of these precipitates is lowered in the presence of complement [Weigle, Maurer & Talmage (218)]. Using S^{35}-labeled complement, Penn *et al.* (219) found that 0.4 to 0.7 per cent of the

protein-bound S^{35} of the guinea pig serum is bound to specific precipitates. Most of the bound activity consists of C'1 [Weigle & Maurer (220); Hawkins & Haurowitz (221)]. Much less S^{35} is bound during the reaction with C'4 or C'2 (221).

Pillemer (222) discovered in the blood serum of different animals a new factor called "properdin." This substance, like C'3, is adsorbed to zymosan at 37°; in contrast to C'3, adsorption occurs also at 15° C. Properdin, in the presence of magnesium ions and C'3, seems to inactivate some of the viruses and to kill certain bacteria and protozoa. Properdin is a lipoglycoprotein which forms about 0.02 per cent of the plasma proteins. Its sedimentation constant is 24 to 30 S, its molecular weight more than one million. It is not adsorbed by antigen-antibody precipitates. Rat serum has high properdin activity, whereas guinea pig serum is poor in properdin. The nature of properdin is controversial. According to Nelson (223) properdin is a complex formed by the combination of antibodies with some of the components of complement.

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CLINICAL BIOCHEMISTRY[1,2]

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I. BILE ACIDS IN BLOOD

Methods of Estimation

For many years, attempts have been made to measure the circulating bile acids of blood, with very variable results. The amounts which have been determined in the many methods have differed widely, and very little of clinical value has emerged. Recently, however, more specific methods have been based on an increased knowledge of the chemistry of these compounds, and there are now several different methods available which produce comparable results.

During the period before ultraviolet-absorption measurements became commonplace, nearly all methods were based upon colour reactions. Pettenkofer, in 1844 (1), described the production of a violet-red colour when cholic acid was treated with concentrated sulphuric acid in the presence of sugar. This reaction was used by Huppert (2), who showed that injected cholate was rapidly removed from the circulation of the dog. Further investigations of the chemistry and specificity of the reaction were reported by Vogel & Dragendorff (3), Mylius (4), Ville (5), Ville & Derrien (6), and Jolles (7, 8). These papers clearly indicated that many substances present in blood or blood extracts could modify or interfere with the colour development in this reaction. Aldrich & Bledsoe (9) thereupon added an extraction process involving a purification by treatment with charcoal and barium hydroxide.

Gregory & Pascoe (10) carried out their important study of this reaction in 1929. They determined the optimum conditions of time and temperature and thus managed to improve the specificity. Further modifications were made by Reinhold & Wilson (11), Schmidt (12), and Irvin, Johnston & Kopala (13). In 1935, Josephson (14) published his extraction procedure, which has since been widely used; the essential part is the use of an ethanolic solution of barium hydroxide, which serves the dual purpose of precipitating protein and absorbing interfering pigments.

The Pettenkofer reaction measures cholic acid or cholates only, i.e., the trihydroxy bile acid. A somewhat similar reaction uses phosphoric acid instead of sulphuric acid, and vanillin is generally added. In this reaction, a rose-pink colour is produced both with cholates and also with the dihydroxy

[1] The survey of the literature pertaining to this review was concluded in April, 1959.

[2] Following recent practice, the authors have made no attempt at complete coverage of this expanding subject. Instead, they have selected, on a personal basis, two topics in which they are interested and experienced.

bile acids (deoxycholic acid and chenodeoxycholic acid). This is a matter of some importance because, as shown by Wootton (15), chenodeoxycholic acid is the major constituent of the bile acids in human bile, and Carey (16) has used paper chromatography of blood extracts to demonstrate that chenodeoxycholic acid is at least as abundant as cholic acid. Abe (17) attempted to develop a technique for the separate determination of dihydroxy and trihydroxy bile acids; the use of this reaction and further modifications of his method were suggested by Kawaguchi (18), Murakami (19), and Häussler (20). A further reaction for the determination of cholate involves treatment with acetic and sulphuric acids, sometimes with the addition of ferric chloride leading to the production of a green colour. This method is usually associated with Szilard (21), and has been used by Katayama (22), Shattuck, Katayama & Killian (23), and Boku & Gon (24).

Using bile acids dissolved in sulphuric acid of various concentrations, Bandow (25), in 1939, published the resultant ultraviolet spectra. These early observations were extended by Eriksson & Sjövall (26, 27), who studied free and conjugated bile acids and obtained satisfactory conditions for quantitative estimations. Kier (28) also confirmed Bandow's work with cholic and deoxycholic acids and proposed a method for their separate determination. Kier's results on normal subjects, however, showed very high values, particularly for dihydroxy acids. As shown by Mosback *et al.* (29), this was probably the result of interference from other substances in the blood extracts which produced non-specific absorption in that part of the spectrum. Other methods based on a similar principle were suggested by Friedman, Byers & Michaelis (30) and Metais, Deiss & Warter (31). Carey (32) and Schiff (33) used ultraviolet absorption combined with Josephson-type extraction and were able to distinguish dihydroxy and trihydroxy bile acids. Other methods of preparing extracts for spectrometry were used by Rudman & Kendall (34) (column chromatography) and Sjövall (35, 36, 37) (paper chromatography).

The green fluorescence produced by heating bile acids with concentrated mineral acids was observed by Jolles (7) and further investigated by Hammarsten (38) and Raue (39). The latter thought that the fluorescence was not affected by many of the compounds known to interfere with the Pettenkofer reaction, such as bilirubin and cholesterol. These observations were not confirmed by Josephson & Swedin (40) and Jenke & Bandow (41), who noted considerable interference from cholesterol, fatty acids, and lecithin. Minibeck (42) attempted to remove these substances by digestion with calcium oxide before measuring fluorescence. A further study of the fluorescent properties of cholic, deoxycholic, and chenodeoxycholic acids was carried out by Turner, Osborn & Wootton (43) and Osborn & Wootton (44). They developed a relatively specific procedure by incorporating the countercurrent distribution developed by Ahrens & Craig (45) and by using paper chromatography in a manner similar to Sjövall (35, 36). In this method also, dihydroxy and trihydroxy bile acids were separately measured.

Normal Values

Most of the authors of methods intended to measure bile acids of the blood have indicated the values obtained in normal subjects. It is instructive to compare the results obtained by the various methods as presented in Table I. Although many results have been reported for dogs, oxen, and other species, Table I is concerned only with the values in human blood (or serum or plasma).

Values in Jaundiced States

There is general agreement in the literature that bile acids in blood are raised above the normal level in jaundiced states; there is much less agreement on the extent of elevation. This probably reflects the imperfect specificity of most colour reactions, since the pigment and lipid components (which have often been implicated as interfering substances) are also raised in jaundice of most types. Thus Jenke & Steinberg (52), whose reported normal values are very low, found values of 2 to 8 mg. 100 ml. Aldrich & Bledsoe (9) reported 7 to 15 mg./100 ml., while Shattuck *et al.* (23) found higher normal values than the preceding authors and obtained results in various forms of jaundice ranging up to 50 mg./100 ml. The normal values reported by Kier have already been mentioned, and, in obstructive jaundice, he described levels of dihydroxy bile acids even higher than his normals. Minibeck (42), and Irvin, Johnston & Kopala (13) agreed that values of up to 20 mg./100 ml. are found in obstructive jaundice; rather lower figures were reported by Sherlock & Walshe (58) and Joppich (60). However, the extensive study of Sherlock and Walshe, who used a Gregory-Pascoe reaction, did not indicate any close relationship between cholic acid level and severity of disease; this was particularly true in cases of cirrhosis.

Sherlock & Walshe's finding has now been explained by the application of spectrophotometric and fluorescent methods that are capable of estimating dihydroxy bile acid (DBA) and trihydroxy bile acid (TBA) separately. Carey (32), calculated the ratio of TBA/DBA in a number of cases. He found about 1.5 mg. of bile acids in healthy persons, with a TBA/DBA ratio of 2.3. In obstructive jaundice, both types of acids were increased, sometimes up to tenfold, but the ratio remained approximately normal. On the other hand, in jaundiced states caused by liver cell damage, the TBA was normal or only slightly raised, but, as he demonstrated, an increase of DBA made the TBA/DBA ratio less than one.

Carey's findings were largely confirmed by Rudman & Kendall (34), who used a rather similar method. With this procedure, people without liver disease showed almost no blood bile acids, but the TBA/DBA ratios that they calculated in case of liver disease were closely similar. Carey (62), using an improved method, extended his previous results and suggested that a TBA/DBA ratio that was less than one was indicative of liver cell damage and might, in some cases, be of great prognostic value. Osborn *et al.* (63)

TABLE I

RESULTS OBTAINED FOR BILE ACIDS OF BLOOD IN NORMAL HUMAN SUBJECTS

Specimen*	Method†	Results‡	Year	Reference
S	Pt	Nil	1924	Herzfeld & Haemmerli (46)
P	Sz	Nil	1926	Perlzweig & Barron (47)
B	Pt	4	1927	Coquellet (48)
B	Pt	2.5–6	1927	Rowntree, Greene & Aldrich (49)
B	Pt	3.1–5.1	1928	Aldrich & Bledsoe (9)
S	Sz	5–12	1928	Shattuck, Katayama & Killian (23)
B	Pt	2.4–3.9	1929	Charlet (50)
B	Fs	80–100	1929	Tashiro (51)
B	Pt	<1	1930	Jenke & Steinberg (52)
S	Pt	0.5	1932	Süllman & Schaub (53)
B	GP	<1	1933	Reinhold & Wilson (11)
S	—	8.8	1933	Kusui (54)
S	PV	Nil	1934	Chabrol *et al.* (55)
B	Pt	0.6–2.2	1935	Josephson (14)
B	UV		1937	Jenke & Bandow (41)
S	Fs	Almost nil	1938	Minibeck (42)
S	PV	Nil	1939	Jenke (56)
S	Fs	0.1–0.9	1940	Gigon & Noverraz (57)
B	GP	0.2–0.6	1944	Irvin, Johnston & Kopala (13)
B	Pt	0.2–3	1948	Sherlock & Walshe (58)
P.S.	UV	35–42	1952	Kier (28)
S	UV	1.7–7.2	1952	Friedman, Byers & Rosenman (59)
S	PV	Nil	1953	Joppich (60)
S	Fs	1.9–3.5	1953	Sobel, Goldberg, & Slater (61)
S	UV	Low	1954	Mosbach *et al.* (29)
S	UV	7.7	1955	Wysocki, Portman, & Mann (66)
S	UV	1.0–2.2	1956	Carey (32)
S	UV	4	1957	Metais, Deiss & Warter (31)
S	Fs	<1	1959	Osborn & Wootton (44)

* Specimen key: B for blood; P for plasma; S for serum.

† Method key: Fs for fluorescence; G.P. for Gregory & Pascoe; Pt for Pettenkofer; PV for Phosphoric Acid-vanillin; Sz for Szilard; UV for ultraviolet absorption after H_2SO_4 treatment.

‡ In mg./100 ml.

adopted a fluorescent method based on a different principle from the preceding but, nevertheless, reported very similar results. They also carried out serial estimations in cases of obstructive and cirrhotic jaundice and showed a close relationship between the clinical state and the blood-bile acid results.

The reason for the alteration in character of the bile acids, as well as the change in the amount, has not yet been proven. The chief dihydroxy bile acid of human bile (15), and probably of human blood (16), is chenodeoxy-

cholic acid (3,7 dihydroxycholanic acid), which only differs from cholic acid by the absence of a 12-hydroxyl group. Bergström & Gloor (64, 65) have demonstrated the presence, in rat liver tissue, of an enzyme capable of carrying out this hydroxylation and also of conjugating enzymes which convert the insoluble organic acids into freely soluble conjugated bile salts. It is now thought that abnormalities in these enzyme systems, or some equivalent in human liver, are responsible for the results that are found in disease. Rudman & Kendall (34) suggested that liver cell damage resulted in defective conjugation and that the unconjugated acids could not be excreted as readily. In this way, they accounted for the high dihydroxy bile acid levels in cirrhosis. They also noted that the trihydroxy/dihydroxy bile acid ratio was higher in the blood of cases of obstructive jaundice than in normal human bile; they attributed this finding to a prolonged exposure of the acids to the hydroxylating enzyme system. Carey (32), on the other hand, explains the high chenodeoxycholic acid levels in cirrhosis by postulating that the side chains of bile acid precursors are degraded to the 24C acid before the deficient hydroxylating enzymes can synthesize the complete cholic acid nucleus.

One of the more distressing symptoms of severe obstructive jaundice is the development of severe pruritus. For many years, this has been attributed, for want of a better explanation, to a high level of circulating bile salts. Recently, evidence of a rather negative nature has been obtained on this point. Carey (62), and Osborn *et al.* (63) noted the relationship between serum bile acids and the symptom. They found that, although cases suffering from pruritus usually had high bile-acid levels, many similar cases with high levels did not itch. Carey artificially produced high blood-cholate concentrations by infusing the pure compound but failed to induce pruritus, while Osborn *et al.* showed that the symptomatic relief offered by steroid therapy was not accompanied by any change in the bile acid level. It is known that the itching can be made worse by the infusion of crude bile salt preparations, but its real cause still remains obscure.

Occasionally one encounters patients who are not jaundiced and who consistently show very high levels of plasma cholesterol. Such hypercholesteraemic people have been investigated by Friedman *et al.* (59), who thought that their circulatory blood-cholate levels were also raised, and by Wysocki *et al.* (66), who said that, on the contrary, a substance resembling chenodeoxycholic acid was present in excess. This point has been re-examined by Osborn & Wootton (44), who found only slight elevation of both trihydroxy and dihydroxy bile acid fractions. They felt that this elevation was completely accounted for by a slight leakage of the high cholesterol content of the blood into the extracts for measurement and that the true bile acid levels of these patients were normal.

Although a considerable amount of work has been done using blood-bile salt estimations in the investigation of liver disease, this test has never become widely used. The reason, clearly, is that the value of the results is not commensurate with the labour involved. In the light of modern work, the

older methods that estimate cholates are of possible use only in obstructive types of jaundice. The degree of obstruction can be assessed as reliably, and very much more easily, by estimating the serum bilirubin, cholesterol, and alkaline phosphatase. On the other hand, liver cell damage appears to cause characteristic changes in the bile acid pattern which are demonstrable by modern methods. In the average case, however, the damage is also reflected in the flocculation tests, the electrophoretic pattern of the serum proteins, and the transaminase values. It appears, then, that only on rare occasions will bile acid analysis assist significantly in the management of the patient.

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II. MAGNESIUM METABOLISM

Analytical Methods

Other than flame photometry, the analytical procedures available for the analysis of magnesium in biological fluids are either cumbersome or inaccurate. The commonly used methods are detailed in Table II.

TABLE II

Procedures for the Analysis of Magnesium in Biological Fluids

Principle	Comment	Reference
Titan yellow	Inaccurate	1, 2
Precipitation as $MgNH_4PO_4$ after removal of calcium as oxalate. Phosphate in precipitate measured by molybdivanadate	Tedious but fairly reliable. Method of choice after flame photometry	3
Colorimetric estimation of Mg-Eriochrome Black T complex after separation of calcium as oxalate	A fair method for plasma. The colour of the dye itself limits the accuracy severely	4
Titration with ethylenediaminetetraacetate after either separation or estimation of calcium	Not suitable for urine	5 to 8
The colour of plasma filtrate added to a solution of Eriochrome Black is matched with a known solution	Not suitable for urine	9

Flame-photometric estimation is made difficult by the much higher excitation potential of magnesium compared with sodium, potassium, and calcium (10). A hot flame, such as oxyhydrogen or oxyacetylene, must be used. The magnesium oxide band at 371 mμ is more easily excited than the atomic line at 285.2 mμ but has been used only at the expense of a cumbersome internal standard procedure (11) to avoid sodium interference or of separate estimation and adjustment of the sample sodium concentration (12). Unfortunately, the flame background at 285.2 mμ is extremely complex, and a further complication is the presence of a sodium line at 285.3 mμ (10). The very narrow band-width that the monochromator must select to isolate the magnesium emission may lead to inadequate sensitivity. Van Fossan *et al.* (13) used an 80 per cent (v/v) acetone mixture as the solvent for this reason. Davis (14) preferred to precipitate magnesium from serum by 8-hydroxyquinoline before measuring the emission at 285.2 mμ. However, MacIntyre & Davidsson (15) were able to estimate magnesium without these added com-

plications by using a specially designed instrument. Further development of this instrument now allows rapid and accurate estimation of magnesium in plasma, urine, and tissues without interference from other inorganic constituents (16). Existing and future commercial instruments will no doubt make this and similar methods easily available in the near future.

Studies with ^{28}Mg

The isotope ^{28}Mg has a half life of 21 hr. and promises to be a very useful tool in the study of magnesium metabolism. Unfortunately, it is rather expensive to obtain carrier-free. It has been used to study gastrointestinal absorption in rabbits (17), sheep (18), and man (19). Distribution of the isotope after parenteral injection has been studied in man, the dog, the rabbit, and the calf (20 to 25).

It is clear from these studies that magnesium readily crosses the cell membrane and is maintained at its high intracellular level by something other than membrane impermeability, possibly by active transport. Furthermore, the isotope penetrates the organs at different rates. Rogers & Mahan (24) showed that, after parenteral injection of ^{28}Mg in the rat, the magnesium in liver, kidney, and heart exchanges within 3 hr. while that in brain, testes, erythrocytes, and skeletal muscle equilibrates much more slowly. These findings are in agreement with those of MacIntyre *et al.* (22, 25), who were also able to show that brain magnesium equilibrates with plasma magnesium by 24 to 48 hr. after injection. At this time, the muscle specific activity is still only about half that of plasma and the bone specific activity only about one-sixth. Both groups consider that intracellular magnesium may exist in more than one form. Magnesium can no longer be looked upon as a stable, inert constituent of the vital organs.

Magnesium Excretion

An adult ingests approximately 20 to 25 m.eq. of magnesium per day. About one-third of this amount is absorbed and excreted in the urine (26, 27). No factor has so far been established to act on magnesium absorption from the gut as vitamin D does on calcium absorption. We are almost equally ignorant about the renal excretion of magnesium.

Chesley & Tepper (28) confirmed earlier findings (29 to 32) that intravenous infusion of magnesium in man increased calcium excretion in the urine. They also showed that the magnesium clearance, corrected for protein binding, increased as a linear function of the serum magnesium and approached the inulin clearance at high plasma levels of magnesium. Unlike earlier workers (30, 31, 32), they found no consistent decrease in urine potassium excretion after magnesium infusion. It was suggested that, normally, almost maximal tubular reabsorption of magnesium is occurring.

Infusion of calcium in man caused a fall in urine magnesium excretion (33), but the plasma magnesium fell to the same extent in this experiment. If conditions are such that no fall in serum magnesium is produced, the urin-

ary excretion of magnesium is then enhanced. It can also be shown that intravenous infusion of strontium results in increased excretion of both magnesium and calcium (34).

Berglund & Forster (35) studied the aglomerular kidney of the marine teleost, *Lophius americanus*. They suggested that a separate transport system for divalent cations must exist to explain their findings. Under their conditions, the divalent cation with the higher excretion rate depressed competitively the transfer of the other ion.

Studying intestinal handling of magnesium, Alcock & MacIntyre (36) found increased calcium absorption in rats on a magnesium-deficient diet and increased magnesium absorption in rats fed a calcium-deficient diet.

The few known facts about magnesium transport by the renal tubule and intestinal mucosa in man are consistent with the existence of a common transport system for the absorption of divalent cations like that proposed for the aglomerular teleost. The proximal tubule is the most likely site in the kidney of such a system (25, 38).

Magnesium Deficiency

Animal studies.—In 1926 Leroy (39) reported that magnesium was essential for growth in mice. A few years later, Kruse, Orent & McCollum (40) described the dramatic effects of magnesium deficiency in the rat: an early stage of peripheral vasodilatation and hyperexcitability followed by a stage of cachexia, muscular weakness, and generalized convulsions. Watchorn & McCance (41) and Tufts & Greenberg (42) confirmed these findings but reported that, save for doubtful changes in muscle and brain, soft tissue magnesium content did not change. However, a marked decline was produced in the magnesium content of bone, teeth, and serum. This failure to produce changes in the soft tissues contrasts with the large decreases in muscle potassium produced by potassium deficiency (43).

In a reinvestigation of experimental magnesium deficiency in the rat, MacIntyre & Davidsson (15) found that there was a progressive fall in muscle magnesium to 78 per cent of the control value after 64 days of deficiency. This contradiction of earlier workers is to be explained by the use of a specific flame-photometric method of analysis and by the expression of the results in terms of dry, fat-free solids to reduce variability (44). In addition, there was a striking fall in muscle potassium to 80 per cent of the control value, although the diet contained ample potassium. The muscle potassium was closely correlated with muscle magnesium ($r = 0.93$). Hypercalcaemia, sometimes very marked, was produced and was accompanied by nephrocalcinosis. This lesion had been consistently found by all workers; histochemical studies have now shown that intracellular calcification occurs in the distal part of the proximal convoluted tubule after as little as nine days of magnesium deficiency (38).

Farm ruminants are prone to a disease characterized by hyperexcitability and associated with a considerable reduction in serum magnesium. The

syndrome occurs in mature lactating cows and is then called "lactation tetany" or "grass staggers," but it also occurs in growing cattle of both sexes and in calves in the first few weeks of life. The annual incidence in suckling calves has been placed as high as 5 per cent (46). The condition is also known to occur in sheep and horses.

Blaxter (45) has recently reviewed hypomagnesaemic disorders of livestock with particular reference to the magnesium content of bone. He analysed muscle tissue from magnesium-deficient calves and concluded that there was no doubt that the intracellular magnesium was unaffected by deficiency. Unfortunately, the concentration of muscle magnesium was expressed in terms of muscle potassium; in view of the results obtained in the rat (15), this would conceal any changes which had occurred. Blaxter calculated that a calf fed a magnesium-deficient diet for 48 days would lose from the bone almost half the magnesium originally present. This figure cannot be accepted because it is based on the assumption that no magnasium is lost from muscle.

In rats (47), the degree of skeletal magnesium depletion varied inversely with the calcium content of the diet; where the rate of bone growth was maintained most of the decline in bone-magnesium concentration was attributable to deposition of magnesium-poor bone rather than to net reabsorption of magnesium. Similar results were obtained by MacIntyre & Davidsson (15) who showed that, under some conditions, bone magnesium does not act as a reservoir but is unavailable to the rest of the body.

Magnesium deficiency in man.—There is now some measure of agreement that the mean normal serum magnesium content is 1.66 m.eq./l (3, 13, 15) with a range from about 1.50 to 1.80 m.eq./l Apparently the healthy body maintains the serum level of serum magnesium just as carefully as it does the level of calcium. There still seems to be complete ignorance as to how it does so, but the hypomagnesaemia sometimes found in primary aldosteronism (59) and primary hyperparathyroidism (56) may provide clues.

Many diseases have been described, usually incorrectly, as being associated with altered serum magnesium. The subject is well reviewed by Wacker & Vallee (12). In recent years, however, a number of reports have appeared of hypomagnesaemia associated with symptoms that disappeared when magnesium was given. The cases fall into two main groups:

(*a*) *Patients maintained on magnesium-free parenteral fluids.*—Flink *et al.* (48, 49) reported gross muscular tremors, athetoid movements, and convulsions in patients with hypomagnesaemia. He also found similar signs in chronic alcoholics, and he claimed that there was an improvement in their condition after parenteral administration of magnesium salts. So many abnormalities were present that ascription to magnesium deficiency of certain of them appears rather arbitrary. Martin *et al.* (50) had previously described 16 patients with hypomagnesaemia in whom symptoms and signs otherwise unexplained were conspicuous by their absence. Hammarsten & Smith (51) successfully treated with parenteral magnesium therapy confu-

sion, tremors, and athetoid movements in a patient with profuse diarrhoea. Randall *et al.* (52) treated 12 cases with psychiatric and neuromuscular symptoms similar to those described above. The patients were severely malnourished and usually had some source of fluid and electrolyte loss. The serum calcium was low in all 12, and the serum magnesium was low in nine. A response to magnesium therapy was claimed. However, the most convincing account of magnesium deficiency in man is that by Card & Marks (53), who describe marked excitement and mental changes in a patient with large losses of fluid and electrolytes from an ileostomy. Treatment with magnesium salts was followed by recovery. Absence in these clinical cases of the hypercalcaemia seen in the rat is explained by co-existence of calcium deficiency as distinct from the animal experiments where calcium intake was carefully maintained (54).

(*b*) *Patients with primary hyperparathyroidism.*—A negative magnesium balance in hyperparathyroidism was reported by Bulger & Gausman (55), but they did not think that this was a direct effect of the hormone. Barnes *et al.* (56) carried out complete balance studies on a patient with hyperparathyroidism in whom the serum magnesium did not exceed 1.20m. eq./l either before or within 20 days after removal of the parathyroid tumour. The magnesium balance, negative preoperatively, became positive after parathyroidectomy. Hypomagnesaemia may occur in primary hyperparathyroidism only after operation, as in the two cases of Potts & Roberts (58); however, in the three cases studied by Agna & Goldsmith (57), there was hypomagnesaemia in the active stage of the disease, a fact which agrees with the findings of Barnes *et al.* (56).

Postoperative hypomagnesaemia may be caused by rapid bone formation, but it is uncertain whether the negative preoperative balance represents a direct effect of the parathyroid hormone. It is interesting that in one of Agna & Goldsmith's cases, the 24 hr. output of magnesium in urine varied between 5 and 11 m.eq./l before the operation despite the presence of a low serum magnesium. It is difficult to explain this finding by an action of parathyroid hormone, because the urine passed six and seven days after removal of the tumour still contained 7–8 m.eq. of magnesium per 24 hr., although the serum level had remained low.

The clinical importance of magnesium deficiency.—There is no acceptable evidence that hypomagnesaemia ever causes true tetany in man, but mental changes, muscular weakness, and, more rarely, convulsions do occur (34, 54). However, whether a persistently low serum magnesium is associated with clinical signs or not, Hess *et al* (38) consider that the rapid kidney damage produced in the experimental animal provides justification for serum magnesium estimations and any consequent magnesium therapy.

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GAS CHROMATOGRAPHY—BIOCHEMICAL APPLICATIONS[1]

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The development of the gas-chromatographic technique by James & Martin (1) has provided tremendous impetus to the general field of chemical analysis. This method has greatly facilitated the rapid and convenient separation, isolation, and quantitation of the individual components of a wide variety of complex mixtures, which heretofore have defied accurate analysis. Today, with rapid advances in technique, quantities of substances —from a fraction of a microgram to grams—can be readily detected and often identified by this procedure alone. The use of gas chromatography in conjunction with additional analytical aids, such as isotopic detectors, infrared, and mass spectrometers, is becoming increasingly prevalent and has enhanced the study of a host of physical and chemical reactions in many fields of science.

Despite the fact that this powerful tool was devised by biochemists and first reported in the *Biochemical Journal*, many recent advances have been made by workers in the petrochemical field. Although a small group of biochemically oriented scientists have also contributed very significantly to further basic developments in the field of gas chromatography, thus far the technique has not been widely applied by the biochemist to problems encountered in research or industrial laboratories. It is for this reason, despite the phenomenal growth of gas chromatography during the past four years, that publications concerned with biochemical applications have been relatively few.

Rather than comment on the multitude of papers discussing various aspects of gas chromatography, this review will concern itself primarily with certain of the more important advances in the technique and, in addition, will survey some of the reports in which the method has been utilized for biochemical problems. An extensive account of the practical and theoretical aspects of gas chromatography may be found in a recent book by Keulemans (2).

Advances in Methodology

The technique of gas-liquid chromatography involves the application of a small sample of a chemical mixture capable of being volatilized onto a column containing an inert support coated with a specific chemical stationary phase. The column, which has a constant flow of an appropriate inert gas

[1] The survey of the literature pertaining to this review was concluded in October, 1959.

passing into it, is maintained at an optimal operating temperature for the specific analysis. The sample components, in the form of a vapor, distribute themselves between the gas phase and the stationary liquid. Differences in the affinity of the stationary liquid for the various component vapors cause them to move at different speeds down the length of the column. The resolved substances emerge from the column as individual "bands" which, upon passing through a suitable detector, give rise to a signal that is amplified and fed into an automatic recording device.

Stationary phases.—The use of gas chromatography was first envisioned by Martin & Synge (3) in 1941 while investigating the concept of liquid-liquid chromatography. At that time they suggested the possibility of obtaining separations of closely related volatile substances by employing as an eluant a permanent gas that passed through silica gel impregnated with a non-volatile liquid phase. The practical application of this theory was left in abeyance until 1952, at which time James & Martin (4) announced the separation of volatile fatty acids from formic to dodecanoic by gas chromatography with a column made up of Celite, a diatomaceous earth, coated with a silicone grease mixed with stearic acid. Nitrogen was the carrier gas, and an integral detection method, which utilized an automatic titration device, was employed in these early days. An additional advance by these workers (5), which served as a stimulus for further developments in the field, was the demonstration of the effect of altering the nature of the stationary phase as a method for achieving separations of various amines. The resolution of a mixture containing ammonia, monomethylamine, trimethylamine, and dimethylamine was readily accomplished on a polar column consisting of 85 per cent 5-ethyl-nonan-2-ol and 15 per cent liquid paraffin on Celite. Decreasing the polarity by increasing the concentration of liquid paraffin relative to hendecanol or the replacement of this type of liquid phase with glycerol, a highly polar material, greatly modified the order of elution of the amines from the column.

Another outstanding achievement in this sphere, which again evolved from the genius of Archer Martin, was the development in 1956 (6) of a new type of detection device, the gas-density balance. This sensitive instrument made possible the measurement of the true density differences between the pure reference carrier gas and a mixture of the column effluent. As a bulk property detection device, it was linear over a wide range; the degree of signal emitted was related simply to the molecular weight of the component band. Using this instrument, James, working with Martin (7), extended the horizons of lipid chemistry by separating fractions of milligram quantities of long chain saturated and unsaturated fatty acid esters extending up to C_{20}. The analyses were performed on columns containing acid-washed Celite impregnated with a thermostable non-polar paraffin grease, Apiezon M, maintained at 200°C. Under these experimental conditions, the unsaturated acid ester emerged from the column before the corresponding saturated ester of the same carbon chain length. Although some columns of this type gave

efficiencies of 3000 or more theoretical plates (as calculated for methyl stearate), the resolution of each fraction of the commonly occurring series of C_{18} acid esters (methyl stearate, oleate, linoleate, and linolenate) was frequently incomplete. Separation of linolenate from linoleate and a tetra- from a penta-unsaturated acid was not possible, even though the presence of a third double bond beyond the 9:10 position in the carbon chain should have, in theory, caused the more rapid movement of this component down the column. Often there was overlapping of the linoleate and oleate bands. In addition, despite the presence of optimal operating conditions, the elution of methyl esters of fatty acids of chain lengths up to 20 carbons from the column frequently took three to four hours. Since it was usually necessary for the columns to be maintained at one temperature, the bands emerging near the termination of the run were often poorly resolved and showed a typical broadening effect. Despite these inherent difficulties, this early work was an outstanding accomplishment and provided a superb starting point for further investigations into the analysis of fatty acids by gas chromatography.

Shortly thereafter, several workers began to explore the possibilities of using other types of liquid phases for the separation of long chain fatty acids in an effort to overcome some of the undesirable features associated with non-polar stationary liquids. In 1958, Orr & Callen (8), working with a commercial polymer "Reoplex 400," and Lipsky & Landowne (9), using an adipate polyester of diethylene glycol, introduced the use of polar stationary phases for this purpose.

A comprehensive study by Lipsky, Landowne & Godet (10) on the further effects of varying the chemical composition of the stationary phase on the resolution of the saturated and unsaturated acid esters showed several striking differences when the results were compared with those noted with the paraffin greases. The individual fatty acid esters that were resolved were eluted more rapidly from the column, thus producing sharper bands. The ethenoid acid esters were retarded and emerged from the column after the corresponding saturated acid ester. The higher the degree of unsaturation within a given carbon chain, the slower was the movement of the band from the column. With the use of a polar phase, it was possible for the first time to separate linoleate from linolenate and a tetraene from a pentaene. Since it seemed apparent that the relative solubility of the sample component vapors in the stationary liquid was to a certain extent dependent upon the degree of polarity of the liquid phase, interesting observations were obtained when this characteristic was altered by increasing or decreasing the number of ester and ether linkages present in the polymer. When the azelate and sebacate polyesters of propylene, butylene, and diethylene glycol were tried as stationary liquids, the same order of elution of the saturated and unsaturated acids was again noted, but, peculiarly, the separation of stearate from oleate did not occur. The development of a variety of more polar high molecular weight (3000 to 30,000) alkyd resins established a pattern which enabled these investigators (10) to correlate further the nature of the chemical com-

position of the liquid phase with the facility and speed with which the long chain acid esters could be resolved. By constructing polymers of ethylene or diethylene glycol and dicarboxylic acids of decreasing carbon chain length (adipate, glutarate, succinate), it became possible to obtain uniformly rapid and complete separation of the straight chain saturated and unsaturated acid esters. Analysis of mixtures of fatty acids extending to C_{26} was now accomplished in less than 60 min. by use of the succinate polyester of diethylene glycol as a liquid phase at 200°C. It was further noted that when some highly polar polymers (particularly those made with glutaric or succinic acids) were used as liquid phases, certain polyunsaturated members of a given chain length were sufficiently retarded to emerge from the column after the succeeding saturated fatty acid ester. Thus, linolenate, the C_{18} triene, appeared after the C_{20}-saturated moiety, methyl arachidate. Similarly, the C_{20} tetraene, methyl arachidonate, emerged after the C_{22}-saturated acid ester, methyl behenate.

Detectors.—As scientists in many different disciplines began to realize fully the vast potential of gas chromatography as an analytical tool, intense interest was manifest in the development of more sensitive detection devices. The early version of the gas-density balance was difficult to produce commercially for technical reasons. Although, unfortunately, it is not sensitive enough for high resolution columns, its precisely linear and completely unequivocal response to all gases and vapors makes it highly desirable as a reference standard wherever really accurate measurements are required. Other more commonly employed bulk property-sensing devices based on a comparison of the changes in the thermal conductivity of a pure reference gas and a gas containing a sample vapor were found to have characteristically undesirable properties. Although suitable for a number of routine analyses, this form of detector, consisting of either a hot filament or a thermistor, is susceptible to small changes in gas flow, pressure, and temperature. Baseline drifts are a common occurrence and often make the detection of trace components difficult. These effects are particularly magnified at high temperatures in the range of 175° to 225°C. Since the best devices based on this principle have sensitivities usually no better than that of the gas-density balance, relatively large sample sizes are required. As noted by Purnell (11), this in turn frequently leads to a decrease in the resolving power of the chromatographic column and prevents the more efficient use of smaller concentrations of stationary liquid on the inert support. It is thought that further increases in sensitivity are not likely to be attained by these instruments, since they suffer from the disadvantage of measuring a small quantity of vapor that is diluted manyfold by the carrier gas stream.

As experience with various types of detectors accumulated, it became increasingly apparent that, in order to achieve substantial increments in sensitivity, techniques would have to be devised that largely avoid this dilution effect on the concentration of sample vapor as it passes through the detector. A logical outgrowth of this type of reasoning resulted in the investigation of

many different types of ionization processes. The electrical discharge detectors of Harley & Pretorius (12) and Ryce & Bryce (13) and the low pressure discharge device of Pitkethly (14) were among the first devices to possess sensitivities several orders of magnitude above that of the conventional thermal conductivity units. The fundamental drawback of these sensing elements was the susceptibility of the hot filament to poisoning by decomposition products of the organic vapors, thus making the long-term response unreliable.

One of the most sensitive and versatile ionization detectors to be devised for use in gas chromatography to date is that described by Lovelock (15). Although an early version of this device bore some physical resemblance to that designed by Deal *et al.* (16), its mode of operation is completely different. The ingenious application of certain fundamental principles of electrical discharges in gases, known to classical physicists for many years, made this device in its present stage of development thousands of times more sensitive than other detectors. The operation of the argon detector depends upon the ionization of organic molecules by collision with metastable argon atoms. As developed for use with packed columns (15), the original device consisted of a small metal ionization chamber to which a high potential was applied. It contained an alpha or beta source of ionizing radiation and had a volume between 3 and 10 ml. When argon was used as the carrier gas and entered the chamber from the chromatographic column, the primary electrons set free in the argon were accelerated by the applied potential to velocities sufficient to excite large numbers of the argon atoms to their metastable state. A trace quantity of an organic vapor emerges from the column and enters the argon-filled ionization chamber, and collisions occur between the vapor and metastable atoms. The ionization potential of the vapor molecule is lower than the excitation potential of the metastable argon atom (11.6 ev). Consequently, there is a transfer of energy from the highly excited but unionized metastable atoms to the organic vapor molecules, which become ionized. The secondary electrons that are produced in the process, when collected at the anode, give rise to an increase in the ionization current, and the increase is related to the vapor concentration. They also generate more metastable atoms, replacing those lost on collision with vapor molecules. The reactions may be depicted as follows:

$$\text{Argon}^\circ + \alpha\text{- or } \beta\text{-rays} \rightarrow \text{Argon}^+ + e^- \text{ (primary)}$$
$$\text{Argon}^\circ + e^- + \text{high voltage} \rightarrow \text{Argon}^* \text{ (metastable state, 11.6 ev)}$$
$$\text{Argon}^* + \text{organic vapor} \rightarrow \text{organic vapor}^+ + e^- \text{ (secondary)} + \text{Argon}^\circ$$
$$e^- \text{ (secondary)} + \text{Argon}^\circ + \text{high voltage} \rightarrow \text{Argon}^*$$
$$e^- \text{ (secondary)} \xrightarrow[\text{to anode}]{} \text{current in amperes.}$$

The detector gives a similar response to many different classes of compounds with molecular weights over 100 and is linear with concentration over a considerable range. Small calibration factors may be required with lower molecular weight substances. In addition to its high sensitivity (10^{-15} moles

per second), this device is relatively insensitive to changes in gas flow, pressure, and temperature. It is also admirably suited to biochemical applications, since only 0.001 per cent of the sample is destroyed in the ionization process. Under these circumstances, virtually the entire sample vapor may be collected intact for further chemical analysis or additional identification procedures such as infrared or mass spectrometry, or radioactivity monitoring devices.

Another sensitive device that has received attention recently is the flame ionization detector of McWilliam & Dewar (17). This instrument, which appears to be a more sophisticated outgrowth of the hydrogen flame combustion detector of Scott (18), is based on the premise that very few ions are formed when a flame of pure hydrogen is burned in air or oxygen. However, when a carbon compound is introduced into the flame, many ions are produced; the proportion of molecules ionized is of the order of one in one million for hydrocarbons and less for highly oxygenated substances. The nature of the process leading to ionization in the flame is still obscure. The temperature of the flame is too low to account simply for the observed extent of ionic dissociation. It seems probable that some cumulative process of ionization is occurring in a manner whereby thermal processes excite molecules to moderate energy levels, whereupon the sharing of the excitation energy resulting from the collision of two excited molecules provides sufficient energy for the ionization of one of them. The sensitivity of this device (10^{-13} gm./ml. gas) is adequate for most needs, although this may have been achieved at a relatively long time constant. Some drawbacks to this type of detector are: (*a*) a sensitivity that varies widely with different classes of compounds; and (*b*) maximum sensitivity can be observed only with equipment capable of handling currents as small as 10^{-14} amperes, thus making rapid response and freedom from spurious effects caused by leakage currents difficult to achieve.

A third device, the radio frequency detector of Karmen & Bowman (19), shows considerable promise and upon further development may possess a degree of sensitivity approaching those mentioned above.

Capillary columns.—Coincident with the development of techniques for the measurement of very small quantities of sample vapor, a new concept in gas chromatography was introduced by Golay (20). In order to avoid the loss of column efficiency associated with the increased gas resistance and subsequent decreases in linear gas velocity provided by the presence of inert support in the regular 0.25 inch internal diameter (I.D.) gas-chromatographic column, he suggested the use of packless columns made by coating the inner surface of long, narrow bore capillary tubing with a thin layer of stationary phase. Such columns, in theory (21), should have efficiencies far exceeding those of packed columns. Although this assumption later proved to be correct, his early attempts with coated capillaries were not very satisfactory because of sample loading difficulties and limitations in the sensitivity

of the microthermal conductivity units employed. Dijkstra & de Goey (22) experienced similar difficulties when they attempted to use these columns for the separation of fatty acids.

Other investigators quickly realized that the efficient use of coated capillary columns would require a much more sensitive detection system than previously used, since it appeared that the maximum load of any single substance that could be applied to the capillary column without affecting the resolving power was in the order of 0.1 μg. One of the few devices known at the time to possess sufficient sensitivity for use with these columns was the micro version of the argon detector developed by Lovelock (23). A modified form of this sensing unit made it possible for Lipsky, Lovelock & Landowne (24) to construct long capillary columns which, for the first time, provided efficiencies that approached 1000 plates per foot of column. Since the rate of gas flow through capillary columns may be as low as 0.25 to 0.50 ml. per minute, it was found that fast-moving components may emerge from the column in a volume as small as a few tenths of a microliter. The effective volume of the modified detector was therefore reduced to one to two microliters in two ways. With an ionization chamber one ml. in volume, a scavenging gas flow of 25 to 50 ml. per minute was swept through the device at a rate sufficient to reduce its time constant to about one second and to prevent the slow diffusion of the sample vapor within the chamber. This expedient, although effective in reducing the volume sufficiently for use with a capillary column, is disadvantageous in that the vapor entering the chamber is greatly diluted and sensitivity is lost in proportion to the total of the scavenging- and capillary-flow rates. This loss of sensitivity was avoided, however, by leading the flow from the capillary column into the chamber by means of a metal tube of small diameter, which also serves as the anode. Under these conditions, the greater part of the electrical field resides within a few diameters of the surface of the tube inlet, and this is the only part of the chamber where the primary electrons are sufficiently accelerated to generate metastable atoms. Thus, when the sample vapor from the column enters the region where there is a high density of metastable atoms, it is able to react and generate secondary electrons before it is appreciably diluted by the scavenging gas flow.

By using this sensing system in conjunction with a 200 foot length of stainless steel capillary tubing with an internal diameter of 0.010 inches and coated with a thin layer of Apiezon L, Lipsky, Landowne & Lovelock (25) obtained efficiencies up to 200,000 theoretical plates and were able to separate certain *cis-trans* fatty acid isomers, e.g., methyl elaidate from methyl oleate, in a relatively short period of time. In order to place very small sample loads onto the column in a reproducible fashion, these workers used a variable stream splitter in the form of a simple T-shaped glass by-pass device, which was isolated from the column compartment and maintained at 300°C. to provide instantaneous volatilization of the high molecular

weight materials. In this manner, a predictable quantity of 99.9 per cent or more of the volatilized sample was vented to the atmosphere. The remainder, approximately 1.0 μg, entered the capillary column.

The capillary chromatographic column possessed a resolving power and a speed of analysis five to ten times greater than that noted with the conventional packed column. An additional increase in resolution may be obtained by employing longer columns (up to 1000 feet) or by using smaller diameter tubing, extending from 0.005 to 0.010 inches I.D. Conversely, more rapid analysis is readily achieved by using shorter (50 to 100 feet) or wider bore columns (0.020 to 0.066 inches I.D.) with only a slight loss in resolution. Indeed, Scott (26) recently presented examples of the wide working range which these columns offer to the analyst. By carefully coating a 1000 foot nylon capillary column of 0.020 inches diameter with dinonyl phthalate, he found that this column gave a maximum efficiency of 750,000 plates for butane, which was eluted shortly after the air peak at room temperature in about 60 min. The efficiency fell to 250,000 plates for *n*-heptane, which emerged from the column in 170 min. At the other extreme, Scott (27), this time working with a 60 foot column of 0.020 inches diameter, was able to resolve the C_4 hydrocarbon isomers in 30 sec. by the use of an automatic sampling valve and by recording the chromatogram on a continuous monitoring cathode-ray screen.

In an effort to express the operating characteristics of these columns in terms of performance at optimal gas flow, Golay (21) suggested the use of an expression called the "Performance Index" (P.I.) in which

$$\text{P.I.} = \frac{\Delta t_x^4 t_a \Delta p}{t_x^3(t_x - 15/16t_a)}$$

Δt_x = width of component band at half height
t_x = retention time of component
t_a = retention time of air peak
Δp = pressure drop across the column in dynes per sq. cm.
P.I. may be expressed in poises (gm. $cm.^{-1}$ $sec.^{-1}$)

In theory, an ideal column should have a performance index of 0.1 poise. In practice, however, many high efficiency columns have had indices at least five to fifteen times this value.

Electron affinity spectroscopy.—An ideal automatic analytical method should possess not only the ability to separate and measure the quantity of components of a mixture but also the means of identifying each component as it is separated. The first two of these requirements are readily satisfied by existing methods of gas chromatography; the third as yet cannot be achieved automatically and frequently requires the laborious method of collecting and identifying by conventional methods each unknown component as it emerges from the chromatographic column. Since the separating ability of chromatographic columns is greatest when the quantity of sample applied is small, the present trend is, therefore, toward the use of high-efficiency coated capillary columns when the sample size is of the order of one microgram or less. When

the total quantity of a mixture is as small as this, the collection and identification of separated components by conventional means is almost impossible. In view of the success of ionization methods for quantitative gas analysis, Lovelock & Lipsky (28) deemed it worthwhile to explore the possibility of developing a highly sensitive ionization technique for qualitative analysis. The mass spectrometer is readily available for this purpose, as was shown by Gohlke (29) and Simmons (30), but it is an expensive and highly sophisticated instrument and at present too sensitive for the latest techniques of gas chromatography.

One of the more promising approaches to the problem appeared to be based on the differences in electron affinity which apparently exist between the principal classes of organic compounds. In the original experiments by Lovelock & Lipsky (28), a simple ionization chamber, which contained an alpha or beta source of ionizing radiation, was maintained at a low applied voltage (0 to 20 volts D.C.). Under these circumstances, when a relatively pure carrier gas (nitrogen, helium, or argon) passes through the device, the negative particles that are formed are all free electrons. These are easily collected at the anode by a small applied potential before they have had time to recombine with the positive ions which are also produced. On the other hand, when an organic vapor is introduced into the system, the free electrons are captured by the vapor molecules to form negative ions which rapidly recombine with positive ions before they can be collected at the prevailing low voltages. The presence of a vapor with an affinity for free electrons is therefore demonstrated by a decrease in "base" current. It was noted that with the great majority of organic compounds the electron affinity is determined principally by the predominant functional group present in the molecule, particularly when this includes some atom other than carbon or hydrogen. The size and configuration of the hydrocarbon moiety appears to have relatively little effect. At specific applied potentials, such classes of compounds as esters, ethers, ketones, halides, alcohols, and hydrocarbons have different capacities to capture free electrons. Under these circumstances it became possible to assign an unknown organic compound to a class after measurement of its electron affinity. The potentialities of the method were illustrated when these investigators (28) clearly indicated the presence of two ketones (3-pentanone and 2-methyl-3-pentanone) in a mixture with 22 hydrocarbons.

Monitoring of radioactive materials.—One of the most important additions to gas chromatography is the development of suitable methods for detecting the presence of radioactive components as they emerge in the column effluent. In this manner, a system is provided for simultaneous determination of mass and radioactive assay of any compound capable of being analyzed by this technique. Applications involving commonly used tracers, such as carbon-14, tritium, phosphorus-32, and sulfur-35, would be endless. Wilzbach & Riesz (31) employed an ion chamber vibrating reed electrometer device in series with a thermal conductivity unit for the monitoring of triti-

ated benzene. Mason, Dutton, & Bair (32) modified a similar system for use at high temperatures (200 to 250°C.) for the analysis of tritium-labeled fatty acid esters.

Utilizing a different principle, Lowe & Moore (33) trapped the vapors emerging from a gas chromatographic column into a circulating liquid phosphor consisting of a solution of 2,5-diphenyl oxazole in xylene. The scintillations excited in the phosphor by the carbon-14-labelled materials were detected with a single tube photomultiplier. The efficiency of such a system was better than 30 per cent. Its disadvantages are: (*a*) the inability to recover the sample component after counting ;(*b*) the necessity of introducing fresh phosphor solutions into the system when the accumulative count rate reaches moderate levels; and (*c*) the maintenance of the monitoring device at a low operating temperature (0 to 4°C.) in close proximity to a heated gas chromatographic detector.

Karmen (34) adapted a somewhat similar approach by condensing his labeled components on siliconized anthracene which served as a scintillator. Under these circumstances, while it is possible to recover the material by extraction, manual trapping of the individual components is still required. Wolfgang & Rowland (35) and Wolfgang & MacKay (36) devised systems utilizing gas proportional counters to monitor automatically the column effluent. When these investigators considered sensitivity, versatility, ease of operation, reliability, and cost, in determining the choice of a counting system, they concluded that, at this stage of development, this type of device is superior to others for all flow applications.

Biochemical Applications

Amino acids.—During the past two years, modest advances have been made in the separation of certain amino acids by gas chromatography. As experience with these substances grew, it became obvious that adequate techniques would have to be devised to convert free amino acids into more volatile compounds. Bier & Teitelbaum (37) surveyed the different chemical forms in which these materials could be analyzed by the gas-chromatographic method. Degradative procedures were most commonly employed. Ninhydrin oxidation yielded aldehydes with one carbon atom less than the parent amino acid. This procedure gave quantitative yields but was applicable only to a few amino acids. Decarboxylation alone, either enzymatically or chemically, produced amines, but yields were poor and varied from compound to compound. Thus far, these investigators have converted 16 different amino acids to either amines or aldehydes.

Bayer (38) tested the applicability of working with the reaction between amino acids and an alkaline hypochlorite solution. Deamination and decarboxylation produced aldehydes of one less carbon atom. The reaction was quantitative, and no side products were formed with aliphatic neutral amino acids. However, acidic or sulfur-containing amino acids gave rise to a complex mixture of volatile substances. Methionine produced mainly propional-

dehyde, which is also formed from aminobutyric acid. Since some amino acids yielded the same products, the usefulness of this technique was lessened. Bayer (38) also separated the methyl esters of amino acids by gas chromatography. Isomers such as norvaline and valine or norleucine and leucine were analyzed on columns containing silicone grease and 10 per cent sodium caproate. More distinct separations were obtained with the N-trifluoroacetyl compounds at 190°C. Tailing was notably reduced, and these substances did not polymerize as readily as the free amino acid methyl esters. Chromatography of the amino acid methyl esters and their N-trifluoroacetyl derivatives on silicone grease containing lithium, sodium, potassium, copper, nickel, and iron caproate was also attempted in an effort to estimate the tendency to form metal complexes with amino groups. Liberti (39), using slightly different procedures, successfully separated alpha amino acids on silicone oil after deamination and conversion to the hydroxy acid methyl esters. Youngs (40), by adopting still another method for the conversion of amino acids to volatile products, analyzed the N-acetylbutyl esters of several amino acids by gas chromatography.

Fatty acids and other lipids.—Since the technique of gas chromatography had its origin in an area that for many years lacked reliable analytical methods, it is logical that, to date, the largest number of biochemical applications involve the analysis of the fatty acid content of a wide variety of tissues and natural products. Beerthuis *et al.* (41) separated certain of the fatty acids found in menhaden oil and *Ximenia* caffra oil on columns containing polar and non-polar stationary phases. The C_{16}-unsaturated methyl ester fraction of menhaden oil was found to contain four different unsaturated acids. Several branched chain acids were also noted. Lignoceric acid (C_{24}) was the highest fatty acid present in the menhaden oil sample. An analysis of the higher molecular weight components of *Ximenia* caffra oil revealed the presence of a homologous series of saturated and monounsaturated acids extending from C_{22} to C_{28}. These investigators also noted differences in the retention volumes of various geometrical isomers of linoleic acid produced by alkali isomerization. A similar but more extensive survey of the fatty acid constituents of menhaden body oil was also made by Stoffel & Ahrens (42). Particular emphasis was placed on the identification of the C_{16}-unsaturated fatty acid fraction, which was obtained by fractional crystallization followed by high vacuum fractional distillation. Countercurrent distribution yielded a pure tetraene, triene, diene, and a monoene. The structures of these acids were then determined by oxidative and reduction ozonolysis, and identification of the fragments was made by reversed phase, paper, and gas chromatography. The last-mentioned technique was an extremely valuable adjunct in providing rapid proof of purity of fractions at various stages in the procedure and in affording critical identification of mono- and dicarboxylic acid fragments formed by ozonolysis.

A study of the fatty acids of sebum from the human forearm by gas chromatography was carried out by James & Wheatley (43). Using a non-

polar liquid phase, it was noted that the principal unsaturated fatty acids contained 14, 16, and 18 carbon atoms. Both odd- and even-numbered straight chain fatty acids were present, as well as two types of branched chain odd-numbered saturated fatty acids. An analogous investigation by Wheatley & James (44) on the composition of the sebum of four types of rodents revealed widely different patterns of fatty acids, thereby suggesting species differentiation. Rattray, Cook & James (45) found the major constituents of the sterol ester fraction of rat livers to contain linoleic, oleic, palmitic, and palmitoleic acids. Another study of the fatty acids in the liver lipids from olive oil and cholesterol fed rats by Rattray, James & Cook (46) demonstrated a great variety of odd- and even-numbered, saturated and unsaturated, and straight and branched chain acids.

The fatty acid composition of human breast milk was determined by Insull & Ahrens (47), who noted that eight saturated and unsaturated components extending from C_{12} to C_{18} constituted 88.4 per cent of the material. The remainder, at least 30 other acids present in small quantities, were odd-numbered and branched chain acids as well as a wide variety of C_{19-29}-unsaturated acids. Further changes in the fatty acid content of human breast milk were correlated with various dietary states by Insull *et al.* (48). During energy equilibrium, milk fat closely resembled dietary fat; however, when insufficient calories were supplied, it resembled the composition of human depot fat. Interestingly, when excess calories were provided to mothers on a "fat-free" diet, the milk showed a significant increase in lauric and myristic acids and a decline in all of the polyenoic acids.

Dole *et al.* (49) fractionated the lipids of the plasma of man into chylomicra, nonesterified acids, triglycerides plus cholesterol esters, and phospholipids. The fatty acid composition of each complex was then determined by gas chromatography. The data showed a consistent pattern for each fraction in a group of normal fasting subjects. No significant alterations in fatty acids were noted during a period of alimentary lipemia.

James, Lovelock & Webb (50) once again employed gas chromatography to study the mechanism of fatty acid synthesis of the cellular components of human blood. After incubation of whole blood with acetate-2-C^{14}, it was noted that the common saturated and unsaturated acids of varying chain lengths were labeled. Although low, but detectable, radioactivity was found in a chromatographic peak which represented linoleic and linolenic acids, this could not be interpreted as conclusive evidence for the synthesis of either or both acids under the given conditions of the experiment.

Since gas chromatography has proven to be much more effective than the best methods of fractional distillation for the separation of complex mixtures, Cason & Tavs (51) applied this technique to a further investigation of fractions of acids (C_{18}, C_{19}) from the lipids of the tubercle bacillus, which had been separated previously by distillation and other techniques. In addition to palmitic, stearic, and tuberculostearic acids, there were also present smaller quantities of three other acids, the esters of which had re-

tention times between palmitic and stearic esters. It was thought that these components probably represented a branched C_{17} acid, the normal C_{17} acid, and a branched C_{18} acid. Ozonolysis of the C_{18} fraction separated by chromatography demonstrated that the unsaturated C_{18} acid was oleic acid rather than vaccenic acid as found in other microorganisms. Fulco & Mead (52) used similar procedures to confirm the presence of 5,8,11-eicosatrienoic acid and its precursor, oleic acid, in male fat-deficient rats given injections of acetate-1-C^{14}. Positive identification of the triene was accomplished by oxidation at the double bonds to yield pelargonic, malonic, and glutaric acids, which, as methyl esters, were readily analyzed by gas chromatography.

Tattrie (53) reported on the use of gas chromatography to determine the positional distribution of the saturated and unsaturated fatty acids of egg lecithin. The saturated acids, myristic, palmitic, and stearic, were found in the α^1 position, while the unsaturated acids, oleic and linoleic acid, occupied the β position of egg lecithin. From these experiments, it was concluded that, since snake venom lecithinase A removes the unsaturated acids from lecithin, the site of hydrolysis must be at the β position of lecithin. Keppler (54) investigated the fatty acid composition of various lipid complexes isolated from the atheromatous plaques found in the aorta of man at postmortem examination. At various stages in the development of the plaque, the phospholipid fraction was found to contain mainly saturated fatty acids. Palmitic acid constituted 35 to 45 per cent and stearic acid 20 to 25 per cent of the saturated acids present. Only small quantities of other C_{12}, C_{14}, C_{16}, and C_{17} saturated acids were noted in these specimens. In cases where the arteriosclerotic process was very advanced, a decrease in the saturated acid content of the phospholipid complex was observed. Analysis of the fatty acids of the sterol esters showed a comparative decrease in palmitic (8 to 25 per cent) and stearic (3 to 10 per cent) acids. The predominant constituents were unsaturated acids. Although the quantities varied for palmitoleic (5 to 10 per cent), oleic (35 to 45 per cent), and linoleic (20 to 30 per cent) acids, these substances were found in increased amounts in subjects with severe arteriosclerotic involvement. It should be mentioned that, although 0 to 10 per cent of the oleic acid fractions isolated from these plaques was in the *trans* form, normal subjects showed the same per cent of *trans* acids when analyses were performed on samples from depot fat, liver, and blood.

Sweeley & Moscatelli (55) described a very interesting gas chromatographic method for the identification and estimation of sphingolipid long chain bases found in animal, human, and plant lipids. Since the sphingolipid bases were polar and non-volatile, it was necessary to convert them to labile derivatives by oxidation with sodium metaperiodate. Cleavage at the double bond or between adjacent amino and hydroxy groups produced fatty aldehydes, which were easily analyzed on gas chromatographic columns containing a highly polar stationary phase (glutaric acid–diethylene glycol polyester) at 180° C. This technique was subsequently applied by

these workers to the analysis of the sphingolipids of human plasma. Sphingosine was the major base component. Smaller quantities of dihydrosphingosine, as well as a significant proportion of a new long chain base, thought tentatively to be dehydrosphingosine, were also noted.

Structural identification of fatty acids.—Considerable information concerning the chemical structure of the individual fatty acid components present in a complex mixture usually has been obtained by comparing the sample chromatogram with that provided when known standards have been analyzed under similar operating conditions. Certain additional data regarding the chain length, the degree of unsaturation, and the degree of branching have been collected by comparing the retention times of a component on two columns containing different classes of liquid phases. James (56) has suggested that this procedure may be best carried out by using Apiezon, a non-polar hydrocarbon, as one stationary liquid and a polar polyester as the other. Indeed, Hawke, Hansen & Shorland (57) collected further chromatographic data concerning the less common naturally occurring *n*-odd-numbered, *iso* and (+)–anteiso fatty acids in this manner. With the use of the slow Apiezon columns, as mentioned earlier in this chapter, the unsaturated members of a series are eluted before the saturated member of the same chain length, although the C_{18} diene and triene and the C_{20} tetraene and pentaene have similar retention times. The polyester columns, however, show differences in the order of elution in that the unsaturated fatty acids, without exception, progressively emerge after their saturated homologues. In certain circumstances, depending upon the degree of polarity of the polyester phases, it has been noted that some polyunsaturated acids may be retained to a point where they emerge close to, coincident with, or after the next higher chain saturated component. James has further stated (56) that the value of non-polar columns in this application rests primarily on the fact that they allow the analyst to distinguish differences in molecular weight and degrees of branching and, to a lesser extent, between *cis* and *trans* isomers. In addition, he thought that polyesters do not differentiate between the position and configuration of the double bond in monoenes, i.e., the distribution coefficients are the same. However, other investigators have found that these assumptions may not apply in all instances. Thus, Keppler (54) noted the ready separation that could be achieved on Apiezon between normal linolenic acid and the isomeric isolinolenic acid; he thought that this could be ascribed to differences in polarities, the latter compound having the greater polarity. Hawke, Hansen, & Shorland (57), using the highly polar liquid phase polydiethylene glycol succinate developed by Lipsky & Landowne (58), reported different retention times for *trans* Δ^9-octadecenoate and *cis* Δ^9-octadecenoate.

Although numerous studies of the fatty acid composition of natural fats have disclosed the presence of a wide assortment of saturated and unsaturated fatty acids that are unlikely to be separated by one type of column packing, it now seems that certain difficulties may be inherent in the combined use of a non-polar and polar stationary liquid for the preliminary

analysis of these complex mixtures. Under these circumstances, it is extremely difficult at times to determine which peaks truly represent the same compound or compounds on two different chromatograms, particularly when many unknown peaks have to be accurately matched together. The collection of individual unknown bands followed by rechromatographing of the sample on another column is an extremely precise and time-consuming task. It seems reasonable to assume, therefore, that a tentative method of identification need not necessarily be restricted to the use of a non-polar and polar phase. If one resorts to employing two polyester phases under conditions which impart different comparative retention times to the sample components, i.e., using polyesters possessing varying degrees of polarity at different temperatures or flow rates, a distinct advantage is to be gained by having the component vapors emerge from the column in the same general sequence. Even though this modification should provide a simpler method of resolving and partially identifying a greater number of acids in a complex mixture, all experienced investigators agree that it is necessary to trap and collect component bands from the column effluent and then subject them to additional chemical procedures for precise clarification of their chemical structure. An elaborate analysis of the fatty acid composition of menhaden body oil by Stoffel & Ahrens (42), who employed extensive chemical and spectroscopic techniques in addition to gas chromatography, is an outstanding example of the type of thorough investigation that may be required before an unequivocal chemical structure can be assigned to an isolated unknown.

Hydrogenation and bromination are common chemical procedures that have been useful in the identification of fatty acids by gas chromatography. A simple micro hydrogenation device that can be utilized in establishing the chain length of an unsaturated acid or the mode of branching of a complex fatty acid has been described by Ogg & Cooper (59). Since some difficulty in completely hydrogenating a polyunsaturated acid often is encountered, investigators have frequently found it necessary to check the extent of the reaction and rehydrogenate with fresh catalyst if necessary. Bromination was employed on a micro scale by James & Martin (7) to confirm the presence or absence of unsaturated fatty acid moieties in samples analyzed by gas chromatography. The newly formed brominated fatty acid complexes, which were much less volatile than the usual saturated acid esters, did not appear when the treated mixture was rechromatographed on columns containing Apiezon at 200°C. It should be noted, however, that Landowne & Lipsky (60) observed the presence of dibrominated fatty acid esters on highly polar polyester columns maintained at their usual operating temperatures. It is not clear at this time whether this can be attributed to a function of column behavior or to dehydrobromination promoted by impurities in the liquid phase. Since the tetra-, hexa-, and octa-brominated fatty acid esters were not eluted from the column, the problem presented itself only when the monoenes were involved.

Oxidative degradation of an unsaturated fatty acid followed by analysis

of the major by-products of the reaction by gas chromatography appears to be the most direct method for establishing the position of the double bonds. Procedures that have been frequently used include ozonization, with reduction of the ozonides to aldehydes by water or hydrogen, and oxidation to acids by silver oxide or hydrogen peroxide. A micro technique for permanganate oxidation has been adapted for use with gas chromatographic fractions by James & Webb (61) and Fulco & Mead (52). Cason & Tavs (51) have also reported on the use of a permanganate-periodate mixture for oxidation. The aldehydes and mono- and dicarboxylic acids that are produced are readily identified by comparing the retention times of a known standard mixture with those of the degradation products formed from the unsaturated fatty acid. It is often difficult to choose which oxidative procedure should be used. The formation of side products resulting from over-oxidation is inherent in each method and often ranges from 5 to 20 per cent of the theoretical quantitative yield. The increasing number of modified methods now in use in different laboratories seems to be indicative of the varying degrees of success that each has with a specific technique. Of paramount importance is the knowledge that the starting material represents a single component. If a high degree of purity is ascertained, then minor products of degradation may be considered to be a result of non-quantitative oxidation rather than of breakdown arising from a small contaminating component present in the fraction under investigation. For reliable quantitative data, confirmation of the results by a second method may be needed.

The discovery of a variety of simple and complex branched saturated and unsaturated fatty acids (and their isomers) in natural fats is now stimulating interest in studies on the elucidation of the chemical structure of such substances. Again, although preliminary data can be gleaned from the usual chromatographic comparisons with the handful of currently available known standards, similar degradative procedures will be required for positive identification of these complex materials. Thus, Agre & Cason (62) developed a method for the chromic acid oxidation of branched chain acids in acetic acid whereby the fragments formed are subsequently analyzed by gas chromatography. In this manner it was possible for these workers to identify eventually the branched acids present in certain lipid fractions isolated from virulent and avirulent strains of tubercle bacilli. Once more it appears that prior knowledge of chain length is necessary in order to "piece together" degradation products correctly. Chain length designation is more difficult as the branching becomes more complex. The branched unsaturated fatty acids that were encountered were hydrogenated and then degraded. Ozonization of an aliquot of the original branched unsaturated compound provided the necessary information concerning the position of the double bonds. Under these circumstances, ozonization was the procedure of choice, since other techniques tend to cleave the molecule at the tertiary carbon centers, making double bond localization very difficult.

Carbohydrates.—The application of gas chromatography to the separa-

tion of carbohydrate derivatives was described for the first time by McInnes *et al.* (63). They observed that the methyl ethers of the methyl glycosides of heat-labile monosaccharides were sufficiently stable and volatile substances to be subjected to analysis by this method. The fully methylated methyl glycopyranosides were chromatographed on Apiezon M columns maintained at 170°C. The two pentose and three hexose derivatives were sufficiently separated from one another to enable quantitative estimation of these components. No structural changes were noted in the sugar molecules when model samples of α- and β-methyl glycosides of 2,3,4-tri-O-methyl-L-arabinose were collected from the column effluent and examined for evidence of decomposition. In a similar manner, Kircher (64) used a variety of stationary liquids (methylated starch, hydroxyethyl cellulose, and guar gum) to effect the resolution of the α- and β-methyl glycosides of glucose, mannose, and xylose at 200°C. The development of additional techniques for forming volatile derivatives, coupled with the use of new stationary liquids and high efficiency capillary columns, will undoubtedly allow the carbohydrate chemist to extend the use of gas chromatography to the analysis of an ever widening number of substances.

Other substances of biochemical interest.—Recalling the observation that pyrolysis of cholesteryl chloride gave rise to a mixture of C_8 hydrocarbons, Cox, High & Jones (65) explored the possibility that analysis of these hydrocarbons by gas chromatography would lead to a new and convenient micro method for the examination of steroid side chains. Fission of the side chain was effected with an acid catalyst, and the major volatile fractions were identified by comparing the retention times with those of known materials on Apiezon L columns at 100°C. Satisfactory results were obtained from alcohols, esters, ketones, and chloro-compounds. Cholesterol and its esters, cholestane, cholestanone, lanostenol and its esters, and lanostanone produced identical chromatographic patterns. Cholesterol and other sterols with C-8 side chains yielded 2-methylheptane and a smaller quantity of hexane. Ergostenol and related compounds with C-9 side chains gave rise to a C_9 paraffin 2,3-dimethyl heptane, but no C_8 hydrocarbon. Under similar conditions, stigmastenol and sitostenol produced a C_{10} paraffin. This simple and rewarding technique should indeed encourage renewed interest in the structural investigations of steroids and tetracyclic triterpenes available only in small quantities.

A more direct approach to the problem of the rapid separation and estimation of steroids was taken by Eglinton *et al.* (66), who attempted to analyze cholestane, 3-methyl cholest-2-ene, and cholestanone on a four foot Apiezon L column operating at temperatures over 200°C. Despite the prolonged period of time required for the elution of these substances from the column (105 to 232 min.) and the obvious lack of high orders of resolution, it was demonstrated once again that complex molecules of low volatility may be subjected to analysis by gas chromatography.

Cornforth & James (67) utilized the gas-chromatography technique to

establish the structure of a streptomycin antagonist found in a crystalline product from *Pseudomonas pyocyanea*. By oxidizing this material and chromatographically comparing the derivatives of the natural product with known synthetic substances, they found the antagonist to be a mixture of alkyl hydroxyquinoline-N-oxides. Similarly, other investigators have been concerned with the identification of complex mixtures derived from natural products. For example, Cason & Harris (68) noted that acetaldehyde was the volatile carbonyl component produced after heating herqueinone, the red pigment from *Penicillium herquei*, with aqueous alkali and converting the neutral material to the oxime, which was then subjected to gas-chromatographic analysis. Carson & Wong (69) applied the technique to the separation and isolation of some aliphatic disulfides and trisulfides in connection with a study of the volatile components of onions. Quin (70), employing gas and paper chromatography and ultraviolet spectroscopy, established that myosmine, nornicotine, anabasine, anatabine, 2,3′-dipyridyl, and cotinine are present in Burley cigarette smoke.

Gas chromatography has also been very conveniently used in the study of gases and other low molecular weight substances. Chmielowski & Isaac (71) demonstrated the biochemical reduction of carbon dioxide to methane by some anaerobic microorganisms in the presence of hydrogen. In this application the principles of gas-solid chromatography were employed in that the various components of the gas mixture were eluted from an absorption column packed with activated carbon. Gil-Av & Herzberg-Minzly (72) have once again shown how important the selection of a stationary phase is to the solution of a particular analytical problem. Realizing that high efficiency of separation can be obtained by the use of reversible complex-forming solutions as the liquid phase, they ingeniously separated nitrogen and oxygen on columns impregnated with blood. Another unique contribution to the field was made by Willis (73), who, by bleeding ethylene in concentrations of 0 to 50 parts per million into the argon carrier stream between the chromatographic column and an "argon" ionization detector, detected 0.5 to 1.0 p.p.m. of hydrogen, oxygen, and methane as they emerged from a Linde 5A molecular sieve column. The recent partial separation of optical isomers by Karagounis & Lippold (74) should be of extreme interest. Using an optically pure stationary phase, diethyl-*d*-tartrate, these investigators resolved certain low molecular weight racemates which under ordinary circumstances could not be separated if the liquid phase were also racemic.

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BIOLOGICAL OXIDATIONS[1,2]

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"Classical" Dehydrogenases

About nine-tenths of the papers dealing with dehydrogenases concern the nine enzymes that are listed in Table I. Only a small fraction of the papers will be discussed in detail.

Comparative enzymology.—Considerable progress may be noted in two different directions. On the one hand, detailed experimental consequences have been drawn from the fact that the classical principles of nomenclature furnish the enzymologist only with the family names of the enzymes. Broad evidence has been presented for the rather surprising phenomenon of the existence of multiple members of the lactic dehydrogenase family, not only in a single organism but even in a single tissue (1 to 5). Here the pioneer work of Meister (6) and Neilands (7) on Straub's preparation of the lactic dehydrogenase of heart muscle has been pursued with considerable success. Markert & Møller (5) have reviewed this subject, covering the whole kingdom of enzymes, and have proposed a new term, "isozyme." This term is applied to different types of enzymes such as hydrolases which show only group specificity as well as to the highly specific enzymes of cell metabolism.

The appearence of multiple forms of one type of enzyme in a single tissue may be closely connected with the existence of different types of cells in the tissue and of different subunits within the cells. Thus, the subject of enzyme localization has received a new stimulus. For example, extramitochondrial and intramitochondrial malic dehydrogenases have been differentiated by kinetic and electrophoretic technique (3, 8, 9).

Until now most of the evidence presented for the existence of enzymes in multiple form stems from electrophoretic and kinetic studies on unpurified extracts. With further progress in preparative investigations, some of the numerous forms described may be found to be artifacts or the effects of nonessential groups attached to the enzyme protein. However, the phenomenon as a whole is now established, and enzymologists have realized that comparative work on multiple forms offers new ways for the study of mechanisms of enzyme action.

[1] This review deals with selected topics on the path of hydrogen in animal cells Most of the literature cited appeared in 1958 and 1959.

[2] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; ATP for adenosinetriphosphate; ATPase for adenosinetriphosphatase, CoA for coenzyme A; DNP for 2,4-dinitrophenol; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form).

TABLE I

DEHYDROGENASES: BIBLIOGRAPHIC REFERENCES

Level of activity, distribution patterns:	Lactic	Malic	Glycerol-1-phosphate	Glyceraldehyde-1-phosphate	Isocitric	Glutamic	Alcohol*	Glucose-6-phosphate
Liver	9, 25, 29; 57 to 60	9, 59, 60	9, 25, 57, 59, 61	9, 25, 57	9, 25, 59, 60	9, 25, 57	25, 57	9, 25, 29; 57 to 59
Muscle	8, 9, 19, 21, 22, 63	8, 9, 22	8, 9; 19 to 22; 61, 63	8, 9, 22	8, 9, 22	8		8, 9, 22
Brain	9	9	9, 61	9		64		9, 27, 64
Blood corpuscles	24, 65		24	24, 65	24			24; 65 to 69
Other tissues and tissue culture	71 to 73		61, 72, 73	72, 73	74			71
Comparative enzymology, multiple forms	1 to 5; 10 to 12; 35, 36, 75	3, 8, 9		37			(Y, L) 35, 36, 76, 77	
Cellular localisation	4, 8, 9, 78	3, 8, 9, 22, 79	8, 22	22, 78	9, 22, 79, 80	8, 9, 79		22
Preparation, test conditions	2, 4, 5, 15, 81	8	8, 61, 62	8	8	82	(L) 83	27
Antibodies	10 to 12			84, 85			(Y) 86	
Active site interactions; Spectral changes, fluorescence	2, 35, 37, 38, 46; 50 to 53; 56, 87	35, 88	35, 40, 121	37, 52, 89		35, 50, 90, 91	(L) 38, 48, 117 (Y) 39, 49, 92, 93 (L, Y) 35, 37, 50	
Kinetics	36, 101, 112			36		91	(L) 48 (Y) 93 (L, Y) 36, 77	
Specificity	106, 108, 116	88	107, 109	37, 110, 120		108	(L) 111 (Y) 39 (L, Y) 76	
Inhibitors	2, 35, 36, 56, 101	35, 88		102 to 104		82, 96	(Y) 93, 105 (L, Y) 36, 77, 82	
Stereospecificity of hydrogen transfer	52		113		114	113, 114	(L, Y) 113	115
Enzyme protein (chemical, physical analysis)	75, 94, 95, 118, 119	88	40, 121	119		91, 96, 97	(L) 48, 83, 100 (Y) 92, 98, 99, 119 (L, Y) 82	
Application in assay for metabolites	127; 137 to 143	127, 128	127, 133; 144 to 147	127, 129, 130	127, 148, 149	131 to 133	102; 122 to 126	127, 130; 134 to 136

* (L) = of liver; (Y) = of yeast.

Last, but not least, the immunological investigations of different enzymes of the lactic dehydrogenase family may be cited (10 to 12), and the opening of new genetic aspects mentioned. Also, a new possibility is offered to clinical diagnosis by means of serum enzyme determination (4; 13 to 16).

Distribution patterns—Distribution patterns that are compiled and biologically evaluated, as conceived by Greenstein (17), constitute another important aspect of comparative enzymology. Unexpected peculiarities in the distribution patterns of some tissues have been reported; for example, in comparison with the jumping muscles, the highly respiring flight muscles of insects contain little if any lactic dehydrogenase together with extremely high levels of glycerol-1-phosphate dehydrogenase (8, 9; 18 to 22). The reverse situation seems to characterize most experimental tumors as well as smooth muscle (9, 23) and enucleated erythrocytes (24, 25). An inborn error of glucose-6-phosphate dehydrogenase in human erythrocytes, detected by Carson *et al.* (26), has found many investigators (cf. Table I). Glucose-6-phosphate dehydrogenase is also included in the outstanding analysis by Lowry's group (27) of enzyme patterns in microgram samples of 10 different regions of rabbit brain. They state that heavily myelinated tracts are low in hexokinase activity and rich in glucose-6-phosphate dehydrogenase, and the data suggest a certain relationship between the activity of the TPN-specific dehydrogenase and the total lipid content of the different regions.

The dynamic state of enzyme patterns in relation to function and to hormonal influences is exemplified by McLean (28) in papers on the mammary gland and by Huggins & Yao (29), on rat liver.

New DPN analogues.—New DPN analogues have been made available by the extraordinary activities of Kaplan's group (30, 31). The authors claim that the topic is "of great importance not only for the study of the properties of the pyridine nucleotide molecule (31 to 34) but should also find an extensive application in future studies concerning the mechanism of action of various dehydrogenases." As a matter of fact this has happened (35 to 46). The comparative point of view has a central position in the field. In their transhydrogenase and diaphorase studies with DPN analogues (43 to 45; 47), Kaplan's group has developed a methodical basis. DPN analogues have also been applied in the extended studies of Van Eys *et al.* (35) on the formation of ternary complexes by several dehydrogenases with the nucleotide and a substrate partner.

Fluorometry.—The general advantages of fluorometry have been summarized by Theorell (48) as an introduction to a detailed report on the pioneer work of his group on liver alcohol dehydrogenase. The "Boyer-Theorell effect," a shift of the maximum to shorter wavelengths combined with an increase in intensity of the fluorescence of bound DPNH as compared to free DPNH, has been shown to occur with yeast alcohol dehydrogenase (49, 50), beef heart lactic dehydrogenases (50, 51, 53), glycerol-1-phosphate dehydrogenase (40), and glutamic dehydrogenase (50), as well as with whole yeast cells (54) and intact mitochondria (55). Furthermore, intensifying and

Application in assay for metabolites	127; 137 to 143	127, 128	127, 133; 144 to 147	127, 129, 130	127, 148, 149	131 to 133	102; 122 to 126	127, 130; 134 to 136

• (L) = of liver; (Y) = of yeast.

quenching effects of substrates and competitive inhibitors have been detected (50), offering new possibilities in the study of binding constants, conformation of the binding sites, etc. (56). Additional information has been drawn from the use of reduced DPNH analogues (38, 46).

As discussed in detail in an outstanding contribution by Velick (52), numerous parameters may be evaluated in fluorometric studies of coenzyme complexes: (*a*) Fluorospectrophotometry deals with the two types of spectra that characterize fluorescence: the activation spectrum (fluorescence intensity as a function of the activation wavelength) and the emission spectrum (fluorescence intensity as a function of the emitted wavelength). (*b*) Not only does the reduced coenzyme act like a fluorescent dye, but the protein partner also has its characteristic activation and emission spectra; thus, in an investigation of the complex, a total of six spectra may be compared. (*c*) The spectral differences between the complex and its components may be interpreted in terms of changes of conformation and group interaction. Furthermore, since both partners are fluorescing dyes, a third factor of considerable interest comes into play: energy transfer from the protein to the bound coenzyme—a phenomenon that contributes information on the special relationships of the bound dihydropyridine ring with respect to certain amino acid residues, mainly tryptophan, of the protein moiety. (*d*) Additional and very important conclusions may be drawn from fluorescence polarimetry by evaluating the depolarization of the emitted radiation of the complex excited by plane-polarized light; first of all, conclusions may be drawn about the degrees of freedom left to the coenzyme in the complex. Velick's paper consists of a comparison of beef heart lactic dehydrogenase and rabbit muscle glyceraldehyde phosphate dehydrogenase—enzymes presenting strong contrasts in spectra as well as in polarization of the fluorescence.

Pyridine Nucleotide Content of Tissues and Mitochondria

The extraction and enzymatic analytical determination of pyridine nucleotides from tissues apparently has still some pitfalls. This is demonstrated by the large discrepancies between the results of different authors on the pyridine nucleotide content of rat liver (124, 133, 148; 150 to 158). Discrepancies may be attributed to variations in any of the three stages of pyridine nucleotide analysis: (*a*) the state of tissue directly before extraction; (*b*) the extraction procedure; and (*c*) the determination of pyridine nucleotides in extracts. In the first step, variations in tissue sampling may influence the redox status of the pyridine nucleotides. The wide variation of extraction procedures reflect the inherent difficulties in the recoveries, especially of the reduced pyridine nucleotides. Conditions for the extraction of pyridine nucleotides from isolated mitochondria have recently been compared and discussed (148).

The determination of pyridine nucleotides by absorption at 340 mμ

(124, 133, 148) may be regarded as more reliable for determining absolute concentrations than are the fluorometric methods. For certain extracts, however, highly sensitive spectrophotometers which measure less than 10^{-10} mole pyridine nucleotides are required (148) in order to compare well with the sensitive fluorometric methods. A differentiating procedure, including twelve fluorescence measurements, has been applied for determination of the four pyridine nucleotide forms (152). In addition to methods that use dehydrogenases, differentiation by nucleotidases (150) and H_2O_2 oxidation (153) has been included in fluorometric determinations.

One recent study of pyridine nucleotide content as a physiological function should be mentioned. In mammary gland the pyridine nucleotide content was shown to adapt to the physiological requirements (28): in lactation the DPN and TPN content increased about eight- to tenfold.

Comparative measurements of the reduced and oxidized pyridine nucleotides in mitochondria from different organs have been reported by Jacobson & Kaplan (151). These data are valuable, particularly with respect to the total pyridine nucleotide content, i.e., the sums of DPN and DPNH and of TPN and TPNH. However, the redox states of the pyridine nucleotides have to be regarded as somewhat fortuitous, since the mitochondria were not maintained in a well-defined functional state (cf. below). Systematic analysis of the pyridine nucleotide content and its molar ratio to the cytochrome-*c* content was recently undertaken (164). These data are compiled together with other scattered data on the pyridine nucleotide content of isolated mitochondria in Table II.

A comparison of pyridine nucleotide content with physiological functions reveals some obvious relations: mitochondria from flight muscle, which should be highly specialized for oxidative energy generation, do not contain TPN; in contrast, liver mitochondria, with their high TPN content, probably participate in the highly active synthetic processes of this organ. Heart and kidney cortex mitochondria, which have a low TPN content, are organs with low synthetic activity as compared with their energy-dispensing activities.

"Mid-Potential" of the DPN-DPNH System

Confronted with the task of presenting an understandable discussion of the oxidation-reduction relations of some DPN-dependent systems, the authors have sought an expression that would distinguish between the two oxidation-reduction potentials E_h and E_0' in the equation

$$E_h = E_0' + \frac{RT}{nF} \ln \frac{[OX]}{[RED]}$$

The term "mid-potential" for E_0' has been found in a paper by Rodkey (166) and we propose to use it for the half-cell potential where [OX] equals [RED] and the specified constant pH does not equal zero.

E_h might then be named simply the "potential" and it is assumed that

TABLE II

PYRIDINE NUCLEOTIDE CONTENT OF ISOLATED MITOCHONDRIA

DPN+DPNH	TPN+TPNH	Reference
μM/gm. Protein		
Liver		
4.4 (DPN)	1.4 (TPN)	(159)
2.7 (total)		(160)
4.6 (DPN)	—	(161)
2.0	4.2	(151)
1.2 to 2.7	1.9 to 5.2	(148)
Heart		
6.4	—	(161)
1.5	0.6	(151)
5.7	0.8	(163)
4.2	1.0	(164)
Kidney		
6.2	1.4	(151)
4.0	0.76	(164)
Brain		
2.1	0.4	(151)
1.3	0.2	(164)
Skeletal Muscle		
3.6	1.1	(165)
Diaphragm		
6.2	—	(165)
Insect flight muscle (Locust)		
2.4 to 4.1	0	(164)
Blowfly		
4.7 (DPN)	—	(162)

both the "mid-potential" and the "potential" are automatically understood as oxidation-reduction potentials, the former being a characteristic constant of the system at a given temperature and a given pH and the latter representing the reducing intensity of the system as it exists in solution, subject to considerable changes because of variation in the proportion of oxidant concentration to reductant concentration.

The effect of temperature on the mid-potential of the DPN-DPNH system was reported in the above-mentioned paper by Rodkey. A mean value for $\Delta E_0'/\Delta T$ at pH 7 of -1.31 mv. per degree has been found by the potentiometric method, which Rodkey developed with xanthine oxidase as catalyst and benzylviologen as mediator.

The new value of the increment was applied and a comparison was then made of the mid-potentials reported from different laboratories and estimated at different temperatures. An unexplained discrepancy is found between the potentiometric value (at 25°), -311 mv., and the values calculated from equilibria and thermal data of dehydrogenase-catalyzed reactions by Burton & Wilson [-320 ± 4 mv (167)] and by Schlenk *et al.* [-326 mv. (168)]. Although the measurements of Rodkey may ultimately be shown to approximate the real thermodynamic value more closely than the others, we

prefer at present to use the value of Burton for practical reasons. On the one hand, a closer relationship exists here to the DPN-metabolite equilibria in which biochemists are interested, and, on the other hand, the mid-potential of Burton has served as a basis for the calculation of numerous other oxidation-reduction potentials and free energy data (cf. 36, 169, 170).

Potential of the Extramitochondrial DPN-DPNH System

The ratios of DPN to DPNH, on the basis of total content, vary from 1 to 3 in liver, kidney, and brain, approximate 25 in skeletal muscle. The subject has been discussed recently (170, 171). This picture will not be essentially changed by a subtraction of the contents of the particulate subunits. Thus, the potential of the extramitochondrial DPN-DPNH system might be assumed to be close to the mid-potential.

However, substantial doubts have recently been voiced (170, 172, 173; discussion in 171) whether conclusions can be drawn from the contents of DPN and DPNH as to the potential of that fraction of the extramitochondrial DPN system that is responsible for the thermodynamic situation and which may be assumed to be represented by the free concentrations. Certainly a considerable amount of the nucleotide is bound to various more or less specific sites and is thus restrained from full activity. Moreover, the binding constants for DPNH of various sites may be considerably higher than for DPN, as is the case with the main pyridino proteins—with the exception of glyceraldehyde phosphate dehydrogenase (references in Table I). This is supported by the observation of an intensive fluorescence of intact cells with a maximum shifted to shorter wavelengths as compared to free DPNH (compare the report on fluorometry above).

An indirect approach to the potential of the DPN-DPNH system is offered by an evaluation of the potentials of those metabolite systems that are connected with the DPN system by the action of dehydrogenases (170, 173). Because of the high activities of some dehydrogenases, the steady state proportions may be not very different from the mass action equilibria. Representative results of such investigations are compiled in Table III.

Concerning the lactate-pyruvate system, it is of considerable interest that the potentials of the blood plasma and the normal resting tissues are very close to each other. Furthermore—especially in liver—the potentials calculated from the contents (on the basis of equilibrium constants and a mid-potential of the DPN-DPNH system of -335 mv.) of three different metabolite systems are very close. These findings have been taken as evidence for the assumption that the potential of the DPN system is also not very different from the metabolite potentials. Certainly, this interpretation is open to criticism from different viewpoints. In the case of the lactate-pyruvate system, however, a more detailed discussion on the basis of the Donnan equation and kinetics has been presented (127). From this, two conclusions have been drawn: (*a*) The potentials of the extramitochondrial DPN systems are strongly coupled to each other by the lactate-pyruvate

TABLE III

POTENTIALS OF DPN-DEPENDENT METABOLITE SYSTEMS AND TOTAL CONTENTS OF LACTATE AND GLYCEROL-1-PHOSPHATE IN DIFFERENT TISSUES OF THE RAT*

	Potentials calculated from total contents†		Total contents‡	
	Lactate—Pyruvate	Dihydroxyacetone-phosphate—Glycerol-1-phosphate	Lactate	Glycerol-1-phosphate
Plasma	−237	—	3.5	—
Liver§	−234	−236	1.1	0.35
Skeletal muscle:				
resting	−242	−230	1.8	0.18
7 sec. tetanus	−242	−256	20	1.4
Heart	−244	−220	1.2	0.11
Brain	−245	−226	2.5	0.12
Lymphosarcoma *in situ*	−254	−235	13	0.09

* See (127, 170).

† Millivolts.

‡ mM per kg. wet weight.

§ Liver: malate-oxalacetate: −235 mv.; total contents corrected for blood and extracellular fluid.

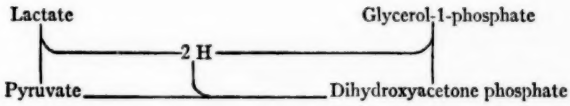
system. (*b*) The average potential of the extramitochondrial DPN-DPNH system may be taken as $E_h = -240$ mv. This corresponds to a DPN/DPNH ratio of the order of 1000, which is far from the values of the total contents mentioned above. The comparatively low reducing intensity of the system involves important consequences for functional considerations with respect to the extramitochondrial oxidation-reduction systems, as well as to the correlations of this system with other compartments of the cell.

OXIDATION-REDUCTION RELATIONS OF L(−)GLYCEROL-1-PHOSPHATE

In the last decade, glycerol-1-phosphate has been discussed mainly with respect to the metabolism of lipids. But now it has its renaissance as an important redox-partner. Investigations on the long-known "glycerol-1-phosphate—pyruvate dismutation" as well as a newly formulated "glycerophosphate cycle" have been reported.

The former system was an essential part of the early glycolytic schemes and later, by competitive inhibition of better experimental evidence, became the *Angärung* system and then disappeared. But the following facts remained: (*a*) high activities of the DPN-specific extramitochondrial glycerol-

1-phosphate dehydrogenase in many tissues, and (*b*) the interesting functional correlation of the reaction catalyzed by this enzyme to the C_3 branch of the Embden-Meyerhof chain with dihydroxyacetone phosphate in a key position:



Thus, the transfer of hydrogen generated by the dehydrogenation of one triosephosphate to another triosephosphate molecule results in the formation of an extra pyruvate, and, consequently the pyruvate level is raised. This was shown to be the case with muscle homogenates by the schools of Embden and of Meyerhof and has recently been "rediscovered" in experiments with insect flight muscles devoid of lactic dehydrogenase (170, 174, 175, 179). Under conditions of inadequate oxygen supply, an extra production of pyruvate caused by a partial consumption of the hydrogen generated in the glycolytic chain by the formation of glycerol-1-phosphate, allows the production of much lactate without a corresponding lowering of the potential. Since the proportion of lactate to pyruvate is about 20, comparatively small amounts of pyruvate are formed. The phenomenon of the rise of lactate levels without a change in the proportion to pyruvate has been shown by Sacks & Morton (176) to take place during tetanic work of striated muscle. Experimental data that explain the effect do so by means of a dismutation of glycerol-1-phosphate to pyruvate (170; compare also Table III).

The mechanism results in a stabilization of the potential of the extramitochondrial DPN system and, hence, of the energetic potential of the extramitochondrial ATP system under the conditions of an inadequate oxygen supply (170, 175).

The absence of glycerol-1-phosphate dehydrogenase in most experimental tumors recently inspired Ciaccio *et al.* (23) to a comparative investigation of the production of lactate and glycerol-1-phosphate in liver and in Novikoff hepatoma under anaerobic conditions. Practically no formation of glycerol-1-phosphate could be demonstrated in the malignant tissue, in contrast to the liver, although an extensive rise of the lactate level occurred in both tissues.

The formulation of the "glycerol-1-phosphate cycle" is another contribution of insect biochemistry. This mechanism has been postulated to effect the transfer of hydrogen from the extramitochondrial DPNH to the respiratory chain (18, 68a) in insect flight muscles. The enzymatic basis is given by the location of the two well-known types of glycerol-1-phosphate dehydrogenases on different sides of the mitochondrial barrier. Hydrogen generated in the Embden-Meyerhof chain and transferred to dihydroxyacetone phosphate by the action of the DPN-specific extramitochondrial enzyme (von Euler-

Baranowski enzyme) may enter the respiratory chain by action of the particulate enzyme (Meyerhof-Green enzyme).

$$\text{Dihydroxyacetone-P} + \text{DPNH}_2 = \underset{\text{(soluble system)}}{\text{glycerol-1-P} + \text{DPN}}$$

$$\text{Glycerol-1-P} + \tfrac{1}{2}\,\text{O}_2 = \underset{\text{(mitochondrial system)}}{\text{dihydroxyacetone-P} + \text{H}_2\text{O}}$$

$$\text{DPNH}_2 + \tfrac{1}{2}\,\text{O}_2 = \text{DPN} + \text{H}_2\text{O}$$

The mechanism is supported by respiratory and spectrophotometric investigations on isolated mitochondria in different states of integrity (177, 178; 180 to 182), determinations of metabolite contents in rest and flight (170, 175), kinetic calculations on the basis of Michaelis constants (179), and the comparison of structural and enzymatic patterns of muscles with different functions (8, 22).

The question has been raised as to whether the glycerol-1-phosphate cycle is also important for the metabolism of vertebrate tissues (9, 170, 183). The soluble dehydrogenase has been shown to be present in high activities in all types of voluntary muscle and in liver, whereas different results have been reported on brain and heart (9, 61). From studies with isolated mitochondria, evidence is available for the existence of relatively high respiratory activities in brain with glycerol-1-phosphate as substrate (183, 184). This is also the case in skeletal muscle (179, 185). The particulate enzyme also has been isolated from these tissues and purified (186, 187, 188). In mitochondria of normal liver the respiratory activity with glycerol-1-phosphate is about one-tenth of the activity with succinate (170, 184) as substrate. However, this proportion changes considerably in favor of the glycerol-1-phosphate oxidation in mitochondria of thyroid-fed rats (189). The content of glycerol-1-phosphate in these tissues has been determined (see Table III) and approximates the content of pyruvate; the only question with respect to the function of the cycle seems to be whether the mitochondria within the tissues exert the same activity on extramitochondrial glycerol-1-phosphate as they do *in vitro*.

A modification of the reaction sequence of the glycerol-1-phosphate cycle in liver attributable to the presence of an intramitochondrial trioseisomerase has been proposed recently (190). On the other hand Bloom (191) has cast doubt on the importance of the glycerophosphate cycle for liver metabolism. From studies on the incorporation of tritium from lactate-2-T and glycerol-2-T into glycogen in liver slices, he concludes that the hydrogen of DPNH formed from glycerol is more extensively used for glycogen synthesis than the one originating from lactate.

If the glycerol enters the pathway by phosphorylation, the action of the glycerol-1-phosphate cycle should introduce glycerol hydrogen into water to a greater extent than lactate hydrogen; thus, the findings seem to be contradictory to the assumption of a highly active cycle. Doubtless the experiments

of Bloom can be appreciated as a valuable approach. However, some other explanations, which are not contradictory to the existence of a glycerol-1-phosphate cycle, seem to be possible. In these experiments, practically all of the tritium introduced with the substrates is recovered as water, only a very small fraction appearing in the glycogen. Differences in the competition between the exchange with water and the incorporation into the glucose molecule could perhaps be attributed to the fact that lactic dehydrogenase transfers hydrogen to the α-position of DPNH, whereas glycerol-1-phosphate dehydrogenase and glyceraldehyde phosphate dehydrogenase are β-specific enzymes. Thus, the DPNH loaded with hydrogen from lactate must be subjected to a shuttle between an α- and β-dehydrogenase before it may be transferred to glyceraldehyde phosphate as suggested by Hoberman (192), whereas the DPNH coming from glycerol-1-phosphate may be accepted immediately. It may be mentioned here that the assumption that the lactic dehydrogenase—DPNH—glyceraldehyde-3-phosphate dehydrogenase complex (113) effects a direct hydrogen transfer is not supported by the fluorometric investigations of Velick (52). Another explanation may be offered by pathways of gluconeogenesis from glycerol which do not involve glycerol-1-phosphate as an intermediate—for example, the one discussed recently by Lamprecht (193). This would assume a twofold dehydrogenation of glycerol at C-1, in the unphosphorylated state, to glycerate and the introduction of the molecule into the pathway of gluconeogenesis at the level of glycerate-2-phosphate.

TPNH as a Hydrogen Donor in Reductive Biosynthesis

A wealth of material has accumulated in recent years for an understanding of the TPN-TPNH system in what may be termed a "high pressure line" of hydrogen functionally connected to numerous biosynthetic pathways. This viewpoint has been conceived by Krebs (194) in connection with thermodynamic considerations of the pathways of glucose formation from C_3-precursors and was substantially supported by the discovery by Glock & McLean of the existence of a high TPNH/TPN ratio in numerous tissues (154). The field may be divided into the following topics: (*a*) energetics, (*b*) biosynthetic pathways, and (*c*) functional co-operation at the cellular level of donor and acceptor system.

Energetics.—Some metabolic systems that may serve to point out the aspects of the energetic situation have been formulated:

Pyruvate + TPNH + DPN + guanosine (or inosine) triphosphate = phosphoenolpyruvate + TPN + DPNH + guanosine (or inosine) diphosphate (194, 195) 1.

L-Xylulose + TPNH + DPN = D-xylulose + TPN + DPNH (196, 197) 2.

Glucose + TPNH + DPN = fructose + TPN + DPNH (198, 199, 200) 3.

D-Glucuronate + TPNH + DPN = L-xylulose + CO_2 + TPN + DPNH (201, 202) 4.

By potentiometric determinations, Rodkey & Donovan (203) have recently obtained new evidence that the mid-potentials of the DPN-DPNH system and the TPN-TPNH system are the same or at least very nearly

equal (204, 205). Therefore, systems in which the chemical potentials of the substrates on both sides are the same, as in Equation 2, or nearly equal, as in Equations 3 and 4, will be practically irreversible if the potentials of the two pyridine nucleotides are considerably unequal; the latter seems to be the case. The potential of the DPN-DPNH system has been mentioned to be in the region of −240 mv. A corresponding evaluation of the potential of the TPN-TPNH system on the basis of metabolite levels has not yet been communicated. However, as recently discussed (170), the concentration of the TPN-specific enzymes—at least in the extramitochondrial compartment—is low, and, hence, the influence of binding by active sites on the proportion calculated from the total contents may be minor.

Another approach is offered by the important fact that the TPN system co-ordinates the metabolite system of the main catabolic pathways which have a mid-potential (at the physiological partial pressure of CO_2), which is considerably more negative than the mid-potential of the TPN-TPNH system. Both the analytically determined TPN/TPNH ratios and the theoretically calculated mid-potentials of the TPN-dependent metabolite systems correspond to an approximate value of $E_h = -400$ mv. for the potential of the extramitochondrial TPN-TPNH system.

On the basis of differences of the potentials of the two pyridine nucleotide systems of −160 mv., the change in free energy associated with the transfer of hydrogen from TPNH to DPN is 7.4 kcal./mole. As a matter of fact, Hers (200) has reported Reaction 3 to be irreversible in experiments with seminal vesicles. The change of the standard free energy associated with Reaction 1 is of the order of 5 to 6 kcal./mole (169; 206 to 208). Therefore, from the energetic viewpoint, the metabolic function of this system in the transformation of pyruvate to phosphoenol pyruvate as suggested by Krebs, has a sound basis. In regard to kinetics, a problem is presented by the relative high Michaelis constant of the malic enzyme for pyruvate (compare Table IV), as compared to the physiological level, which might be assumed from the tissue contents.

Biosynthetic pathways.—In the first column of Table IV, a review is attempted on some of the related enzymes and enzyme systems that have been discovered, isolated, and characterized in connection with the biosynthetic function of TPNH. The biosynthetic pathways under discussion are named briefly in the middle column. Some older papers have been included in the table, but the historical aspect is by no means covered.

Functional co-operation.—The relationship between the hydrogen-donating pathway of carbohydrate catabolism and the hydrogen-consuming system has been the subject of interesting investigations. Here the application of differentially labelled glucose has entered a new stage. The extended studies of the ratio of $^{14}CO_2$ formed from glucose-1-^{14}C and glucose-6-^{14}C with respect to the relative contribution of the direct oxidative pathway have been reviewed recently by Dickens (209). In the numerous papers referred to subsequently the "C-1 minus C-6" values of the CO_2 production have been used

TABLE IV

Enzymes and Pathways in TPNH-Dependent Biosynthesis*

Properties (and localisation) of the enzymes	Biosynthetic pathways concerned	Remarks
Malic enzyme (extramitochondrial) (237 to 239)	Glycogen, citric cycle intermediates from 3 carbon precursors (194, 195, 218, 250)	K_m values for pyruvate [M]: malic enzyme: 2×10^{-2} (237) pyruvic kinase: 1×10^{-3} (207)
Glutamic dehydrogenase (mitochondria, references in Table I)	Glutamic acid and derivatives (251, 252)	*In vitro:* DPNH and TPNH facultative (108)
Glutathione disulfide reductase (115, 240) (wide occurrence in cytoplasm of mammalian tissues) specific for TPNH and GSSG (240)	Maintenance of reduced state of glutathione (66, 253); cooperation with glutathione peroxidase of erythrocytes? (254)	Low levels of GSH in erythrocytes with deficiency of glucose-6-phosphate dehydrogenase (references in Table I)
α,β-Unsaturated acyl CoA reductase (cytoplasmatic rat liver) (microsomal, pig liver) (241, 242)	Fatty acid synthesis (225, 255)	
Acetyl CoA-reducing system (extramitochondrial, pigeon liver, two enzymes resolved) (243 to 245)	$8\ \text{AcCoA} + 16\ \text{ATP} + 16\ \text{TPNH (or DPNH)} \xrightleftharpoons{CO,\ Mn^{+}}$ palmitic acid $+ 16\ \text{ADP} + 16\ P_i^{+} + 16\ \text{TPN (or DPN)}^{+} + 8\ \text{CoA}$ (slower rates with DPNH as H-donor)	
Palmityl CoA reductase (microsomal, rat brain) (246)	Sphingosine (256)	
β-Hydroxy-β-methyl-glutaryl-CoA reductase (yeast extract) (247)	Mevalonic acid; isoprenoids (257)	Coenzyme requirements of cholesterol synthesis by liver microsomes (267 to 270)
D-Glucuronate reductase (10,000 gm. supernatant, liver, kidney) (248, 249)	L-Xylulose and ascorbic acid from glucose or inositol (201, 202; 258 to 262; 264 to 266)	Distribution in different animal species (271)
L-Xylulose reductase (mitochondrial, guinea pig liver) (196, 197)	D-Xylulose ("glucoronic acid-xylulose-cycle") (197, 263)	
Aldose reductase (supernatant seminal vesicle) (198, 272)	Glucose → fructose transformation (199, 200)	
Folic acid reductases (chicken liver acetone powder) (273 to 275)	FH_4 postulated as H donor for the reduction of formaldehyde in thymidine synthesis in *E. coli* (284)	Equilibrium favours reduced heterocycles (in contrast to TPN-linked dehydrogenation of the hydroxymethyl group) (288, 289)
"Mixed function oxidases" requiring TPNH and oxygen (276):		Reaction mechanism discussed in (278)
Biotransformation of drugs (microsomal systems) (277 to 279)	Oxidative deamination; aromatic hydroxylation; dealkylation (285, 286); side chain oxidation; oxidation of phosphorothionates (287)	Cofactor requirement (290)
Phenylalanine hydroxylase (combined system of sheep and rat liver enzyme) (280)	Tyrosine formation	Replacement of TPNH and sheep liver enzyme by H_4-folate or synthetic H_4-peteridines; inhibition by folic acid antagonists. (290a)
Kynurenine hydroxylase (222, 281)	Nicotinic acid; xanthurenic acid	
β-Hydroxylation of steroids (beef adrenal gland supernatant plus particulate fraction) (281)	Corticosterone; cortisol	Compare also (290b)
Aromatisation of steroids (placenta) (282)	Estrogen hormones	
Unsaturation of fatty acids (supernatant plus particulate fraction of yeast) (283)	Unsaturated fatty acids	

* See (171, 250)

for estimation of the extent of TPN reduction by the direct oxidative pathway. From experiments with various tissues and under different metabolic influences, the important conclusion arises, that the rate of generation of TPNH by the direct oxidative pathway is strongly influenced by the rate of TPNH consumption. This has been suggested by Kinoshita (210) on the basis of experiments with corneal epithelium under anaerobic conditions. The "C-1 minus C-6" technique was used also by Brin and co-workers (211, 214) who proved finally that the methylene blue stimulation of erythrocyte respiration originates from a mediator function of the dye in the reoxidation of TPNH. It may be mentioned that science is here confronted with a historical event: the methylene blue effect demonstrated personally by E. S. G. Barron (212) to Otto Warburg initiated the investigations (213) that led directly to the discovery of glucose-6-phosphate dehydrogenase and to the isolation of TPN.

The examination of the influence of redox mediators on the TPNH production by the direct oxidation pathway of glucose-6-phosphate has been carried on by Hastings' group (215) with investigations on liver slices from normal and alloxan diabetic rats and on hemidiaphragms. The liver slices, when incubated in a pyocyanine-containing medium, showed a more than twofold increase in the incorporation into CO_2 of glucose-1-^{14}C and a sharp simultaneous reduction in the incorporation of carbon from glucose into glycogen and fatty acids, as compared to the control. The same was essentially true with slices from diabetic liver, at considerable lower rates. Wenner and co-workers (216, 217) applied the "C-1 minus C-6" technique to the glucose catabolism of ascites tumor cells and investigated the influence of various agents with interesting results: (*a*) only minor influences of respiratory inhibitors (cyanide, azide, amytal) on the C-1 minus C-6 values of the CO_2 production are observed in the CO_2 production from glucose carbon; (*b*) the uncoupling agent dicumarol stimulated the oxidation of carbon atoms, except those derived by the direct oxidative pathway; (*c*) the reduction of the C-1 minus C-6 oxidation under the influence of uncoupling agents, as well as under the influence of iodoacetate, may be directly correlated to a decrease of the level of pyruvate. Therefore, further evidence is offered by these investigations for a marked influence of the hydrogen-accepting system on the rate of the direct oxidative pathway, and, probably, for a functional explanation of the discrepancies that have been found in the evolution of CO_2 from glucose-1-^{14}C in experiments with liver slices and with the intact animal by injection of differentially labelled glucose (229).

In their functional relationships as hydrogen consumers of the biosynthetic processes presented in Table IV, the formation of glycogen and fat have been studied in detail.

Liver.—The 3-carbon branch of anabolism and catabolism has been studied *in vivo* and *in vitro* by addition of 2-C^{14} alanine (218) and pyruvate (195), and of deuterolactate (192, 219). The isotope distribution in glutamate and glycogen confirms the reductive carboxylation of pyruvate as an essential

metabolic pathway in liver. In starving rats it is followed preferentially.

Adipose tissue.—Winegrad & Renold (220) have studied the fate of specifically labelled glucose with respect to oxidation and incorporation into fatty acids in paired epididymal fat pads. Three to four times as much C-1 as C-6 is oxidized to CO_2, whereas twice as much C-6 as C-1 is converted to fatty acids. Addition of insulin *in vitro* increases the C-1 oxidation another six- to sevenfold, while the C-6 oxidation is only slightly increased. At the same time, the incorporation of C-1 and C-6 into fat increases more than an order of magnitude. Glucose is required for the synthesis of fat from acetate or pyruvate. The ratio of C-1 to C-2 in CO_2 evolution is 0.92/0.24 without insulin and 6.36/0.33 with insulin; for incorporation into fatty acids the ratio is 0.19/0.38 and 3.06/6.31, respectively. Fatty tissue appears to be an interesting model for both the magnitude of stimulation of C-1 oxidation and the insulin effect. The authors demonstrate thereby that the C-1 to C-6 relationships in oxidation and lipogenesis are not completely explainable on the basis of present concepts of metabolic pathways.

Mammary gland.—Extensive studies of glucose metabolism in various stages of mammary gland lactation cycle were presented by McLean (28). The quotient of C-1 to C-6 oxidized to CO_2 increases from 1.5 on the twentieth day of pregnancy to 15 at the height of lactation, and from 6 at the end of lactation to 2.2 on the third day of involution (in rats). Oxidation-reduction mediators stimulate the oxidation of C-1 preferentially. Uncoupling agents increase the oxidation of C-6. The effect of insulin on C-1 oxidation increases during lactation. These results were correlated with detailed studies of the variations in enzyme and metabolite patterns.

Diabetes.—The well-known fact that lipogenesis is disturbed in spontaneous and experimental diabetes, as well as in starvation, has been discussed in connection with the fact that TPNH plays a role in synthesis of cholesterol as well as of fatty acids (221, 225, 226). This aspect has been discussed in detail by Siperstein & Fagan (224), as the experimental evidence on homogenates of fasted and alloxan diabetic rats has demonstrated the reversal of lipogenic defects through initiation of TPNH generation. In this way, the assumption of a second metabolic block in insulin lack becomes superfluous.

However, the above-mentioned works provide some considerations against this theory since it is shown that the rate-limiting factor lies in TPNH consumption. Cahill *et al.* (215) have described a doubling of CO_2 evolution from C-1 in liver slices from alloxan diabetic rats by the influence of pyocanin. The extraordinarily great insulin effect on adipose tissue points also to a stimulation of C-1 oxidation by increased TPNH consumption. The fact that in starved rats, in contrast to fed ones, pyruvate is transformed almost exclusively to dicarboxylic acids contraindicates a defect in TPNH regeneration (192, 218, 219). Shaw & Tapley (227) have analyzed about equal total contents of oxalacetate in normal and alloxan diabetic rats. Abraham *et al.* have shown from experiments on homogenized alloxan diabetic liver

that no defect in the TPNH regeneration occurs (70). In following up these researches, Matthes *et al.* (228) have shown a reduction of the TPN-specific microsomal α,β-unsaturated acyl-CoA reductase in alloxan diabetic liver to 30 per cent of normal.

Transhydrogenation.—If it develops that in most tissues the steady state oxidation-reduction potential of the TPN system is significantly more negative than that of the DPN systems and the correspondingly higher hydrogen pressure drives biosyntheses, then transhydrogenation signifies a loss of cellular free energy. However, the metabolic organization of many tissues may be adjusted in such a way that biosynthetic processes have only subordinate significance. This may occur in heart muscle where, most observers maintain (however cf. 80), the isocitric dehydrogenase is TPN-specific.

The often-described systems of transhydrogenation may be classified in the following way: (*a*) biosynthetic transhydrogenation like that in reactions 1 to 4 (p 679) in which the gradient of hydrogen pressure between TPN- and DPN-systems drives in one direction; (*b*) the simultaneous presence of a TPN-specific and a DPN-specific transhydrogenase for the same substrate inside a compartment in which this substrate can diffuse; (*c*) a dehydrogenase with incomplete nucleotide specificity and its substrate [examples of this are discussed by Navazio *et al.* (106), Holzer & Schneider (108), and Kinoshita & Futtermann (120a)]; (*d*) true dehydrogenases [the particulate enzyme of Kaplan *et al.* (205) is an example; Hagerman & Villee (230) have recently claimed that the estrogen-sensitive principle in placenta represents a soluble true transhydrogenase].

The problem of estrogen-dependent transhydrogenation, raised by Talalay & Williams-Ashman (231), has stimulated many studies, but with contradictory results (232 to 236). It now appears clear that the DPN-specific isocitric dehydrogenase of placenta can be removed from the list of enzymes. The nature of the estrogen-sensitive transhydrogenase, however, must still be clarified.

Hydrogen Pathway in Mitochondria

Redox status of mitochondrial pyridine nucleotide systems.—The redox status of pyridine nucleotides is a sensitive indicator of the functional state of mitochondria. Since the classical studies of Chance & Williams (291), the recording of absorption changes at pyridine nucleotide-sensitive wavelengths in mitochondria has been extended to other mitochondria (148, 164, 170, 292, 293). Interference in the absorption changes by other components, particularly cytochromes, should be considered. Thus the apparent shift of the absorption maximum of mitochondrial pyridine nucleotides to a shorter wavelength (294) was later explained as cytochrome-*c* interference (182, 292). In aerobic difference spectra (e.g., controlled minus active state[3]) with-

[3] Controlled state (148) [State 4 (291)]: substrate present, slow respiration, no phosphate acceptor. Active state (State 3): phosphorylation in the presence of substrate and phosphate acceptor.

out major interference, the absorption maximum at 340 mμ is the same for the mitochondrial bound as for the free pyridine nucleotides.

Spectrophotometric and analytical methods were combined to avoid these difficulties and to determine the contributions of DPNH and TPNH to the absorption changes in intact mitochondria (148, 164, 170). Under control of the double-beam spectrophotometer, mitochondria were brought into definite functional states and the pyridine nucleotides extracted.

With respect to the DPN-systems, the following results were obtained. In the active state with a relatively high turnover of DPN, the steady state reduction is very small or not detectable. In the controlled state, however the greatest part of DPN appears to be blocked from the reducing action of DPN-specific substrates; only in liver mitochondria does the DPNH-DPN ratio approach one. It has been suggested that in mitochondria DPN is bound or compartmentalized and is thus only partially available to the DPN-specific dehydrogenases (296). The inhibition could also be caused by a compound DPN-1, as has been assumed for the mechanism of oxidative phosphorylation (297). In fact, the detection of DPN-I and TPN-I has been claimed on the basis of enzymatic analysis (298); however, these experimental results could not be confirmed (148). A formation of TPN-I, in the same manner as of DPN-I would be improbable, since TPN appears not to be involved in oxidative phosphorylation.

A high degree of reduction of mitochondrial DPN in the controlled state is only observed on the addition of substrates that do not involve DPN for oxidation, such as succinate in rat tissues and glycerol-1-phosphate in flight muscle. This surprising phenomenon has now been established by enzymatic analysis as a general reaction in mitochondria from various organs and species (164). In this context, the phenomenon was interpreted and discussed in detail as a reversal of oxidative phosphorylation, implying a reduction of DPN by flavoprotein, as originally proposed by Krebs & Kornberg (299) in another context. Similar conclusions had been reached by Chance (296) on the basis of spectrophotometric studies on kidney mitrochondria. The underlying mechanism was described by the following reaction sequence (170):

$$\underset{\text{(or succinate)}}{\text{Glycerol-1-phosphate}} + A \rightarrow AH_2 + \underset{\text{(or fumarate)}}{\text{Dioxyacetone phosphate}}$$

$$AH_2 + \sim X \rightarrow AH_2X$$

$$AH_2X + DPN^+ \rightarrow A + DPNH + X + H^+$$

A = flavin, $\sim X$ = energy-rich intermediate or ATP

The relation between the redox status of the DPN and TPN system could be most clearly demonstrated on liver mitochondria (148). Thus, under nearly all conditions tested, TPN was reduced to a higher extent than DPN. This difference is probably maintained by functioning oxidative phosphorylation. In uncoupled mitochondria both systems may show the same redox status. Kinetically, changes in the redox status of the TPN are relatively slow compared to the rapid redox changes of the DPN system. Therefore, a rate-

limiting transhydrogenase that links both systems is proposed. Only the DPN appears to be directly linked to the respiratory chain and oxidative phosphorylation.

Fluorometry was applied to mitochondria (55), tissues (302), and single cells (301) as a new tool for determining the redox status of pyridine nucleotides. Reduced pyridine nucleotide binding was demonstrated in mitochondria in a manner similar to that previously used with isolated dehydrogenases. The question remains open as to whether this demonstrates the postulated DPNH-I intermediate of oxidative phosphorylation or other types of binding. Chance & Thorell (301) applied microfluorometry to single spermatozoa. They were able to measure separately the fluorescence of the cytoplasm and of the *nebenkern* (merged mitochondria) of these cells. The fluorescence was shown to originate from pyridine nucleotides. An important result of these experiments is the existence of compartmentalized pyridine nucleotides, since in the cytoplasm DPN is mostly reduced and in the mitochondria mostly oxidized. This finding gives further support to the theory that the mitochondrial membrane *in vivo* is impermeable to reduced pyridine nucleotide (170).

The binding of pyridine nucleotide in mitochondria was also investigated by studying the release or incorporation of pyridine nucleotides to mitochondria (163, 300). The incorporation of DPN apparently requires the presence of ATP but appears not to be an active process. Thus, it can also be observed in the presence of ethylenediaminetetraacetate or triphosphate. The incorporated DPN is presumably bound to a great extent to unspecific sites, since the amount incorporated can be two to three times that of intact mitochondria (300). The pyridine nucleotides of mitochondria are (163, 304) only released if they are in the oxidized state. Ethylenediaminetetraacetate and substrates, however, can also stabilize intramitochondrial binding of oxidized pyridine nucleotides. There are indications for some very tightly bound DPN, since after fragmentation of mitochondria a small fraction of the original DPN remains attached to the submitochondrial particles (305, 306).

The redox state of DPN and TPN in liver mitochondria was shown to be influenced by the specificity of the substrates (148). With isocitrate a relatively higher steady state reduction of TPN was measured than with β-hydroxybutyrate, indicating TPN-linked isocitrate oxidation with transhydrogenase as a rate-limiting step. This pathway has been also suggested by determination of the related enzymatic activities (307). The reported intracellular localisation of a high transhydrogenase activity in mitochondria further supports the reaction sequence of isocitrate oxidation (308).

Steroid effects.—Biological effective steroids have been shown to inhibit at concentrations as low as 10^{-5} the respiration of DPN-specific substrates in actively phosphorylating mitochondria (309). No inhibition of succinate respiration was observed. Under the steroid influence the reduction of both the mitochondrial DPN and TPN was increased to the level of the controlled (non-phosphorylating) state (148, 170). This and direct spectro-

photometric evidence suggest a point of action on the respiratory chain between DPN and cytochrome-*b* (310). A possible physiological significance was inferred from the close relation between the *in vitro* to *in vivo* effectiveness of various steroids. Most effective were corticosterone, deoxycorticosterone, aldosterone, testosterone, progesterone, estradiol, etc.; less effective was cortisol; ineffective were cortisone, tetrahydrocortisone, etc. Inhibition by steroid hormones has been reported on the oxidation of DPNH of various mitochondrial and microsomal systems (311, 312).

Non-pyridine nucleotide-linked oxidations.—Various aspects of the mitochondrial glycerol-1-phosphate oxidation have recently been studied. Its relation to the respiratory chain was spectrophotometrically investigated in flight muscle mitochondria (170, 180). DPN and a greater part of the mitochondrial flavin were found to be only indirectly involved in glycerol-1-phosphate oxidation. A smaller portion of flavin could possibly form the prosthetic group of the dehydrogenase (182). Brain mitochondria proved a relatively rich mammalian source for glycerol-1-phosphate dehydrogenase (183). From this material the enzyme was solubilized by phospholipase and purified (188, 313). Also on brain mitochondria a hydrogen transfer between glycerol-1-phosphate and fumarate below the antimycin-A level was demonstrated (188, 316). The distribution of glycerol-1-phosphate oxidase activity in mitochondria from various rat organs showed highest activity in brain and skeletal muscle and lowest activity in heart (184). Another non-pyridine nucleotide-linked mitochondrial enzyme, choline dehydrogenase, was isolated from liver mitochondria (319 to 321). Here also, the relation to succinic dehydrogenase was studied on intact mitochondria (335). The reported DPN dependence of choline oxidation by mitochondria was shown to be attributable to further DPN-linked betaine aldehyde oxidation (314, 321).

A physiological role has now been assigned to diaphorase (322, 323), which has been identified with lipoic dehydrogenase. The diaphorase functions in hydrogen transfer of ketoglutarate oxidation between lipoic acid and DPN (324). This enzyme had been independently identified as a flavoprotein with a relatively negative redox potential (325 to 327). A newly isolated "lipoflavoprotein" was reported to be the mother particle of the classical diaphorase and Mahler's cytochrome-*c* reductase, which are assumed to have lost the lipid part (315). This lipoflavoprotein has no lipoic reductase activity. The intracellular distribution of diaphorase activity was reported to show highest activity in the cytoplasm. This fraction has equal DPNH and TPNH specificity, in contrast to the mitochondrial fraction (303; cf. also 295). A related finding is the separation of two types of diaphorases from liver mitochondria by diethylaminoethyl cellulose; the two types differ in their specificity to DPNH and TPNH (43).

Respiratory Chain

Cytochrome pattern.—In Table V the absolute and relative cytochrome contents of various preparations and intact cells have been collected. Only the major, well-established cytochromes of animal tissue have been in-

cluded. No differentiation between cytochromes *c* and c_1 was possible, since these data have been obtained by absorption photometry at room temperature.

In a comparative investigation (164), the highest cytochrome content of mitochondria was found in flight muscle mitochondria. This is in accord with the enormous oxidative metabolism of the intact flight muscle, the highest of all animal tissues. The second highest, but with considerably lower value, is the cytochrome concentration of heart mitochondria. Generally in mitochondria, more cytochrome-$c+c_1$ is found than cytochrome-*a*. Apparently the major part of cytochrome-*c* has been washed out in submitochondrial particles.

A correlation between cytochrome-*c* content and the morphological fine structure in three different muscles of *Locusta migratoria* (Linnaeus) has been demonstrated. In the three types of muscle, the cytochrome-*c* content is proportional to the mitochondrial volume of the tissue and to certain related enzymes (22). Estabrook (328) estimated the cytochrome-c_1 content by low temperature absorption and by first extracting the cytochrome-*c*. In liver mitochondria cytochrome-c_1 amounts to 35 per cent of the total cytochrome-$c+c_1$ content, whereas, surprisingly, no cytochrome-c_1 could be detected in insect flight muscle mitochondria (181).

Electron pathway of the respiratory chain.—Slater (329) has written a review article on the constitution of the respiratory chain, covering the literature until the beginning of 1957. Green (330 to 333) has published several articles summarizing this groups' work on fragmented beef heart mitochondria. These articles also discuss hypotheses on the structure of the respiratory chain. Some of these viewpoints are considered below.

Slater (329) concluded that the electron transport particle of Green's group is identical with the Keilin-Hartree preparations from heart muscle. The hypothesis (332) that separate cytochrome chains exist for DPNH and succinate oxidation has been criticized by several authors (188, 329, 334, 335). The separation of the DPNH and succinate oxidase activities should be explained as a selective inactivation of these functions in the electron transport particle. A subfragment with a high DPNH and low succinate-oxidase, which was obtained by mechanical disruption of heart mitochondria should be mentioned in this connection (336, 337). Kimura & Singer (335), in an interesting study on the competition between succinate and choline oxidation by liver mitochondria, conclude that the electron pathway between cytochrome-c_1 and c_3 is common to the oxidation of both substrates.

The discussion of these aspects is closely connected with considerations of the relation of cytochrome-*b* to the respiratory chain. In a review article (329), Slater maintains that cytochrome-*b* participates in the electron transfer of succinate oxidation but not of DPNH oxidation and that both pathways merge onto the same cytochrome chain after the cytochrome-*b* stage. Evidence from Green's groups, however, would indicate that there is no participation of cytochrome-*b* in succinate oxidation (338). A new fragment

TABLE V

CYTOCHROME PATTERN OF THE RESPIRATORY CHAIN

Preparation	Concentration* Cytochrome-*a*	Relative Amounts of Cytochromes (*a*=1)		Reference
		c+c_1	*b*	
Mitochondria				
Rat liver	0.2	1.7	0.9	(291)
	0.16	1.6	—	(164, 165)
Rat heart	0.6	1.7	0.9	(292)
	0.3	1.4	—	(164, 165)
Rat kidney-cortex	0.21	1.9	—	(164, 165)
Flight muscle (*Musca domestica* Linnaeus)	—	1.7	0.5	(180)
Flight muscle [*Locusta migratoria* (Linnaeus)]	0.53	1.45	0.95	(182)
Ascites tumor	0.2	3.1	1.0	(293)
Yeast	0.15	1.4	1.5	(414)
Submitochondrial particles				
Rat liver (digitonin)	0.23	1.8	0.7	(306)
Beef heart (electron transport particles)	0.76	0.85	0.91	(317)
Phosphorylating particles	0.73	0.55	0.93	(421)
Intact Cells				
	10^{-9} moles/gm cells (or gm. wet wt.)			
Ascites	7.0	2.2	0.3	(414)
Yeast	20.0	2.5	1.4	(414)
Frog sartorius	2 to 3	1.8	0.6	(414)

* 10^{-6} moles/gm. protein.

that is active in the electron transfer from succinate to cytochrome-*a* contains only succinate dehydrogenase, cytochromes-*c* and c_1, but not cytochrome-*b*. Another fragment containing the succinate dehydrogenase—

cytochrome-*b* complex, which is active in the reduction of cytochrome-*c* and coenzyme Q, shows only a slow reduction of cytochrome-*b* (339). Chance (340) finds that on addition of certain inhibitors (antimycin-A, ethyl hydroxyquinoline N-oxide) to heart muscle preparations cytochrome-*b* can be reduced rapidly, in contrast to the slow reduction observed on addition of cyanide or on transition to the anaerobic state. Possibly these inhibitors cause cytochrome-*b* to be reincorporated into the respiratory chain, corresponding to the hypothesis that cytochrome-*b* is in the phosphorylating but not in uncoupled respiratory chain. Similar effects of the inhibitors, as observed by Jackson & Lightbrown (341), are interpreted as an inhibition of the auto-oxidation of cytochrome-*b*, which is believed in all these cases to be in the pathway of DPNH and succinate oxidation.

Thorn (342 to 344) has made a profound study of the influence of unspecific reducing and oxidizing agents on the redox status of the cytochrome and succinic oxidase. The dependence of succinic oxidase activity on phosphate could be explained by protection of sulfhydryl groups from oxidation. The influence of peroxidase-H_2O_2 on a side-linked system of possible physiological significance has also been investigated by Martin & Stotz (345). Peroxidase acts on cytochrome-c_1, competing with the cytochrome oxidase. A reconstitution of the succinic oxidase system by combining succinic dehydrogenase with alkaline-treated heart muscle preparations has been reported by Keilin & King (346).

Colpa-Boonstra & Holton (347) have determined the redox potential of cytochrome-*b* with these substrates and find $E_h = 75$ mv. According to Chance (340), however, the redox potential of cytochrome-*b* cannot be determined by the substrate couple fumarate-succinate, since cytochrome-*b* apparently is not in equilibrium with these substances. This is shown by the effect of inhibitors, which increase the velocity of reduction by succinate and decrease the velocity of oxidation by fumarate.

Cytochrome oxidase.—In a new attempt to separate cytochrome-*a* from a_3, it was found that even after electrophoresis of cytochrome oxidase preparations no change in the ratio of the absorption at 445 mμ (a_3) to 605 mμ (a) can be detected (348). In another study, cytochrome oxidase was shown to be reducible by DPN-cytochrome-*c* reductase (349) without the mediation of cytochrome-*c* itself; however, these experiments scarcely give a basis for physiological conclusions. Wainio *et al.* (350) discuss again the function of copper in cytochrome oxidase as a possible oxygen acceptor or electron carrier, or even of cytochrome-*a* as a cuproporphyrin. A relation between the copper content and cytochrome oxidase activity and, further, a molar ratio of 1 to 1.5 between copper and hemin content are reported. Evidence for the formation of a cytochrome-*a* oxygen compound has been claimed by Okunuki *et al.* (351) and Sekuzu *et al.* (352). The absorption maximum of cytochrome-*a* shows a slight wavelength difference between cytochrome-*a* oxidized by oxygen or by ferricyanide. Similar results and interpretations are published by Minnaert (353). Activation of cytochrome oxidase by lipids has been demonstrated (354 to 356).

*Cytochromes-*c *and* c_1. The amino acid compositions of cytochrome-*c* from horse heart and yeast were investigated (357, 358). The small observed differences in the chemical composition of the two cytochrome-*c* types are reflected in a slight shift between the α-maxima of the reduced forms: 550.0 mμ (heart) and 549.5 mμ (yeast). On cytochrome-*c* that has been digested by pepsin some data have been obtained on the structure of the core by amino acid analysis and formation of intramolecular hemochromogens. The molar extinction of highly purified oxidized and reduced cytochrome-*c* between 220 mμ and 600 mμ has been determined with high accuracy (359). These data are also valuable for difference spectrophotometry of cytochrome-*c*-containing particles.

A synthesis of cytochrome-*c* and incorporation of labelled amino acids has been demonstrated on isolated heart mitochondria (360). The synthesis is prevented by uncoupling oxidative phosphorylation. The cytochrome-*c* content in various rat organs is reported to decrease during the development of a Walker carcinoma (361). A lipophilic form of cytochrome-*c* can be isolated from the "electron-transport particle" (362); by the action of phospholipases or butanol, it can be converted to the known water-soluble cytochrome-*c*. The lipophilic form is assumed to be the native form of this cytochrome and to function in the respiratory chain (333). This would imply that the lipid part is split off in some manner during the common extraction procedure for cytochrome-*c*. Cytochrome-c_1 can be isolated from submitochondrial fragments in a highly purified form with deoxycholate and butanol (363). The isolated cytochrome with a molecular weight of 380,000 is supposedly a polymer of units, each of which has a molecular weight of 70,000 and one hemin (333).

Vitamin K in electron transport and oxidative phosphorylation.—Several substances of which the structures are characterized by a quinone part and a polyisoprenoid chain gain increasing attention as possible cofactors of electron transport and oxidative phosphorylation. The review on this subject will include also some papers that appeared shortly before the last two years. Martius (364, 365) has given further support for the proposed role of vitamin K in electron transport by isolating a phylloquinone reductase. This possible role of vitamin K was also the subject of a comparison of the kinetics of menadione (vitamin K_3) and DPNH oxidation in non-phosphorylating heart muscle preparations (366). It was concluded that menadione does not participate in electron transport between DPNH and cytochrome-*b*. An activating effect of vitamin K_1 and K_2, but not of menadione, on electron transport could be shown on the succinate oxidase of heart muscle preparations, which had been inactivated by isooctane treatment (367 to 371). Menadione further increases respiration only in activated preparations, presumably by an extra pathway of non-enzymatic oxidation of menadione. In these studies, the activation by vitamin K_1 could be duplicated by vitamin E and ubiquinone. Several other substances that lacked a quinone ring but contained an isoprenoid chain of varying lengths proved to be activators.

Whereas the role of vitamin K in electron transport is much disputed,

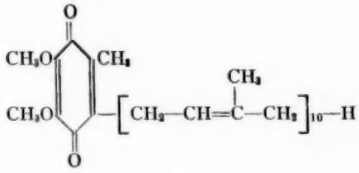
its role is oxidative phosphorylation is increasingly supported by evidence of both an enzymatic and chemical nature. A specific requirement of vitamin K in oxidative phosphorylation has been developed by ultraviolet-irradiation of various preparations. This method proved to be valuable in studying the vitamin K requirement of isolated systems, as well as in studies that included vitamin-K deficiency by dietary means (372). After irradiation of bacterial extracts (373, 374) and of isolated liver mitochondria (375 to 377), oxidative phosphorylation was greatly decreased and could be reactivated only by vitamin K, but not with menadione or vitamin E. The relatively small irradiation inhibition of succinate-linked phosphorylation was reactivated by cytochrome-*c* alone, but not by vitamin K (376). Therefore, oxidative phosphorylation and electron transport between DPNH and cytochrome-*b*, preceding the amytal sensitive step, are further regarded as the specific site of action of vitamin K. A specific inactivation of the DPN-sensitive ATPase by irradiation has been quoted as evidence for the action of vitamin K in the phosphate transfer mechanism of oxidative phosphorylation [(378); cf. below].

A possible chemical reaction of vitamin K in phosphate transfer has been formulated (379). Thus, a phosphate ester of the reduced form of vitamin K should, on oxidation, transfer phosphate onto ADP to form ATP. In fact, this phosphate transfer could be demonstrated on the oxidation of the phosphate ester of dihydromenadione by bromine (380). Also quantum mechanical considerations give support to an activation of the phosphate ester on oxidation from the aromatic to quinoid electron system (381).

Vitamin E.—The role of vitamin E in electron transport was investigated on isooctane-extracted preparations of DPN-cytochrome-*c* reductase (370; 382 to 385). The reactivation proved to be unspecific, since several lipids could function as reactivators instead of vitamin E. However, a specific requirement for vitamin E developed on aging of DPN-cytochrome-*c* reductase. The unspecific effect of lipids was interpreted as a mobilization of endogenous vitamin E, which thus is made available to the electron transport. These arguments are based on the finding that by isooctane alone only about 10 per cent of vitamin E can be extracted, whereas in combination with lipids a considerably higher percentage is involved. These reactivations are attributed specifically to vitamin E. In contrast, experiments were described indicating that the inactivation is an inhibition effect of absorbed isooctane, which is removed from the inhibiting sites by vitamin E. The finding that lyophilisation or centrifugation can reactivate the isooctane-extracted preparations is a further indication of the inhibiting effect of isooctane (386 to 388). As another explanation for the specific requirement of vitamin E in aged preparation, its role as an antioxidant in preventing peroxidation of lipids essential for electron transport function (389) was offered. The participation of tocopherol in electon transport still appears improbable.

Ubiquinone-coenzyme Q (Ubi-Q).—Reviews have been published on the present status of research on ubiquinone (390) and coenzyme Q (330, 333).

It is almost certain that ubiquinone and coenzyme Q, studied independently by two major research groups, are preparations derived from the same *in vivo* group of compounds. We shall use in this article the common abbreviation Ubi-Q for this group of substances. Both research groups have studied the chemical nature of Ubi-Q (390 to 393). In collaboration with industrial laboratories, both groups arrived independently and simultaneously at identical final structures (394, 395) for mammalian Ubi-Q;



The Ubi-Q that was first isolated from animal tissue and mitochondria was shown to have 10 isoprenoid groups. However, it was soon recognized that Ubi-Q homologues exist in various microorganisms (396 to 398), which vary in the length of the isoprenoid chain. The structure of Ubi-Q has been confirmed by synthesis of Ubi-Q derivatives and total synthesis of the Ubi-Q of yeast, which contains six isoprenoid residues (396). A detailed study on the distribution of Ubi-Q in animal tissues, microorganisms and plants showed its ubiquitous distribution (398). In mammalian tissues only Ubi-Q with 10 residues is found. Homologues with smaller chain lengths occur in insects and microorganisms. The coenzyme Q content of mitochondria is of particular importance for the assumed role for Ubi-Q in electron transport. Heart mitochondria = 3.5 μM/gm. protein; liver mitochondria = 1.7 μM/gm. protein (398). It should be emphasized that DPN is found in these mitochondria at about the same concentration (164). The ratio Ubi-Q/cytochrome-*c* in mitochondria can be calculated to be eight (heart) and six (liver). In the electron transport particle and heart muscle preparations, about 3 to 4 μM Ubi-Q/gm. protein are found (399 to 401).

The influence of coenzyme Q on isooctane-inactivated particles shows a complicated pattern. In isooctane-inactivated preparations of succinate-cytochrome-*c* reductase, Ubi-Q proved to be only one of several substances, i.e., vitamin K and tocopherol, that act by the isoprenoid chain, as is discussed above (371). There appears to be a problem similar to that with tocopherol in extracting all of the Ubi-Q which is present (399). Only by extraction with the combination of deoxycholate and isooctane a requirement for Ubi-Q and also for cytochrome-*c* appears. Ubi-Q was thought to act only in the electron transport chain of succinic oxidase, but not of DPNH oxidase (399). Adding cytochrome-*c* to inactivated preparations was thought to form a link between both chains, thus bridging the gap caused by Ubi-Q-extraction. A better procedure for developing a specific requirement for Ubi-Q appears to be acetone extraction (402). The succinate

respiration of acetone-extracted mitochondria could only be reactivated by Ubi-Q and cytochrome-*c*.

Data more relevant for the role of Ubi-Q were obtained by following the oxidation and reduction of coenzyme Q in mitochondria and submitochondrial particles. The functional role for Ubi-Q in electron transport was suggested by the early demonstration of oxidation and reduction of endogenous or added Ubi-Q in electron transport particles (403). Also with mitochondria, Ubi-Q could be partially reduced by succinate and DPN-linked substrates (404). A nearly complete reduction was effected by addition of cyanide. The reduction of Ubi-Q was inhibited by amytal with DPN-linked substrate, but not with succinate. Antimycin-A does not inhibit the reduction of endogenous and added Ubi-Q but inhibits its oxidation (405, 406). Some contrary reports on the action of antimycin-A (404) apparently could not be further confirmed (333). Thus, the point of action of Ubi-Q was shown to be between the point of amytal and antimycin-A inhibition. Specifically, in succinate oxidation Ubi-Q is assumed to form the link between the succinic dehydrogenase flavoprotein and cytochrome-c_1 (338). Cytochrome-*b* is thought to not function in this electron transport chain. A lipid that contains a relative high amount of Ubi-Q could be isolated (407 to 409). This lipid is considered to have an important function in the electron transport between the succinic dehydrogenase flavoprotein and cytochrome-*c* (338).

Attempts have been made to obtain kinetic support for the function of Ubi-Q in electron transport. A velocity of oxidation and reduction consistent with the over-all electron transport has been claimed (404); however, no exact measurements are yet available. On the other hand, on heart muscle preparation a velocity of reduction of only 10 to 25 per cent for added Ubi-Q and 50 per cent for endogenous Ubi-Q in relation to the over-all respiration has been reported (400).

A relation between the redox state of Ubi-Q and the functional state for mitochondria has been studied with the result that Ubi-Q as well as DPNH can be oxidized on addition of ADP to these mitochondria (410). In this connection, phosphate was observed to promote the reduction of DPNH and Ubi-Q. This was thought to indicate a generation of energy-rich intermediates on phosphate addition. A possible alternative explanation is that, under the special conditions employed, the mitochondrial suspensions became anaerobic, and thus reduction of DPN and Ubi-Q was produced. In favour of this interpretation is the reported reduction of DPN and Ubi-Q on aging of the mitochondria.

Oxidative Phosphorylation

Respiratory control.—The significance of the respiratory control as a sensitive criterion of physiological intactness of isolated mitochondria has been stressed (182, 297, 411). The first step of respiratory control, i.e., acceleration of respiration by addition of phosphate acceptor, appears not to be as

sensitive as the second step, i.e., deceleration of phosphorylation after consumption of ADP, since in the presence of too high an ATPase activity control could still be observed at the first but not at the second step. For quantitative description of respiratory control, therefore, the ratio between respiration with and after ADP consumption is to be preferred. Other phenomena are shown to be clearly related to respiratory control and therefore may also be taken as criteria of physiological intactness (164, 182). An example is the glycerol-1-phosphate induced reduction of mitochondrial DPN. Respiratory control as observed on isolated mitochondria has recently been discussed as a regulatory principle *in vivo*, particularily with respect to insect flight muscle. Respiration there can change a hundred fold between the resting and active states. Since no respiratory control was shown in phosphorylating mitochondria from *Musca domestica*, other mechanisms of regulation were considered (177, 180). From the flight muscle of *Locusta migratoria*, however, mitochondria could be obtained that have respiratory control with all substrates tested (170, 182). The function of this basic regulatory principle for insect flight muscle must thus be considered similar to that of other animal tissues. A fundamentally different control mechanism could be the observed inhibition or activation of glycerol-1-phosphate respiration by complex agents or divalent metal ions (177, 68a, 412). The suggested exclusive control of flight muscle respiration by this principle, however, was disputed. It cannot be applied to tricarboxylic acid cycle substrates, which could be shown to sustain high respiration in isolated mitochondria (175). Respiratory control phenomena *in vivo* could be demonstrated on ascites tumor and yeast cell suspensions on addition of intracellular ADP-releasing substrates (318; 413 to 417). This interpretation was confirmed by the isolation of mitochondria from ascites tumor cells showing respiratory control (293).

Magnesium.—Magnesium exerts an important, apparently paradoxical, effect on the respiratory control. In liver mitochondria, magnesium stabilizes or reconstitutes respiratory control of loosely coupled preparations (411). However, magnesium can abolish respiratory control in mitochondria from heart, kidney, and brain (184, 418), apparently because of the high magnesium-activated ATPase of these mitochondria.

Evidence that magnesium is a cofactor of oxidative phosphorylation can be derived from experiments on mitochondrial fragments. The requirement for magnesium could be clearly demonstrated on fragments obtained from sonicated liver mitochondria (419). The specific function of magnesium in binding a soluble protein cofactor has been suggested, since the cofactor is effective only in the presence of magnesium (420, 421). The requirements for this cofactor arise only if ethylenediaminetetracetate is added during fragmentation of mitochondria. A magnesium requirement has now been demonstrated also on digitonin-treated particles (422, 423). The previous inability to show a magnesium requirement is to be attributed to the higher

content of endogenous magnesium of the digitonin particles used (424, 425). A requirement for magnesium in the ATP-ADP^{32} exchange could recently be shown with a solubilized enzyme preparation (426).

Endogenous coupling and uncoupling factors.—Several investigations are devoted to an endogenous principle of uncoupling. "Mitochrome" (427) was separated by isooctane extraction into a heat-stable uncoupling factor and an ineffective hemin, which is formed on aging of cytochrome preparations (428, 429). A similar uncoupling substance can be obtained on aging of submitochondrial fragments (430). This factor presumably consists of fatty acids, of which the uncoupling effect is already known. The protective effect of serum albumin on oxidative phosphorylation, particularly as shown on flight muscle mitochondria, has been demonstrated to be a binding of the endogenous uncoupling agent (431, 432). This effect is -SH group dependent. A related, remarkable finding is the purification from human plasma of a coupling factor that can even reverse uncoupling by DNP (433). The possible bearing on the uncoupling mechanism of DNP must await further studies. Another factor, liberated from mitochondrial fragments, that abolishes respiratory control but not oxidative phosphorylation has been described (434, 435). This factor might be effective in mitochondria from hyperthyroid rats or insect flight muscle, which both lack respiratory control. It must be questioned whether basic or only quantitative differences are shown by the uncoupling and respiratory control-releasing factors.

Possibly closely related to the uncoupling agents are soluble protein fractions, which can promote the coupling of oxidative phosphorylation. They have been obtained from mechanically fragmented beef heart mitochondria (436). Probably another type of coupling is effected by heat-stable substances released from mitochondrial fragments (437, 438). This cofactor can be replaced by coenzyme A in coupling oxidative phosphorylation with substrates (DPNH, succinate, D-β-hydroxybutyrate), that do not require coenzyme A for dehydrogenation.

Mechanism of oxidative phosphorylation.—Various aspects of the reaction sequence between energy conservation at the respiratory chain and the formation of ATP have been further investigated. The following reaction sequence (439, 440) is adopted by most authors with respect to the consecution of the phosphate transfer. However, more or even less intermediate steps may be included in the various mechanisms proposed.

$$\text{Electron transfer} \rightarrow C \sim X \rightarrow C + X \qquad 1.$$

$$C \sim X + P \leftrightarrow C + X \sim P \qquad 2.$$

$$X \sim P + ADP \leftrightarrow X + ATP \qquad 3.$$

Thus, first an energy-rich phosphate intermediate (and not an energy-rich ADP intermediate) is formed and then transfers phosphate to ADP to form ATP. Several types of evidence are now available to make this choice

between the two alternative sequences of phosphate transfer in oxidative phosphorylation.

Using H_2O^{18}, a split between the terminal P-O phosphate ester bond was shown on hydrolysis of ATP (441, 442). This can be explained only by formation of the intermediate P∼X, thus excluding the intermediate ADP∼X. Conclusive evidence was further obtained from ATP-P^{32} and ATP-ADP^{32} exchange. Thus, the ATP-ADP^{32} exchange proved on several grounds to be a part of the ATP-P^{32} exchange (426, 439): only the ATP-ADP^{32} exchange is DNP-sensitive. The ATP-ADP^{32} exchange is more rapid than the ATP-P^{32} exchange (439); the ATP-P^{32} exchange is ADP-dependent; however, the ATP-ADP^{32} exchange is not influenced by phosphate. The solubilisation from mitochondria of an enzyme that catalyzes only the ATP-ADP^{32} exchange was announced (424, 426). On the other hand, an apparently different ATP-ADP^{32} exchange-catalyzing preparation, which needs inorganic phosphate, was described (443). How these apparently contradictory findings can be reconciled must await further data.

Investigations on the primary point of energy conservation have been based on the action of DNP in exchange reactions and ATPase. It has been shown and emphasized that the DNP-activated ATPase, together with both phosphate exchange reactions, are a part of the oxidative phosphorylation process (439, 441, 442, 444). This has been explained as the result of the DNP-catalysing reaction 1, thus causing reversal of reactions 2 and 3. Kinetic considerations of the phosphate exchange favour the action of DNP on the decay of a non-phosphorylated intermediate (Reaction 1). Also on the basis of O^{18} exchange studies, DNP was considered to act on reaction 1 (441) but, at variance with this, X∼P decay has also been implicated as a DNP reaction site (442).

Diaphorase-flavin has been considered as a point of DNP action (445). Thus, agents specifically influencing flavin, such as amytal, flavin inhibitors, and redox dyes, were shown to be inhibitors of the P^{32}-ATP exchange and of the DNP-activated ATPase (444; 446 to 448). A further support on inactivation of the DNP-activated ATPase by visible light has recently been reported (449). A similar differentiating inactivation of the DNP-activated ATPase by ultraviolet irradiation, in contrast to the magnesium-activated ATPase, has also been described (450) and has been interpreted as being caused by destruction of vitamin K or related quinones, which might be active in the phosphate transfer.

Cytochrome-b_5 and Microsomal Enzyme System of DPN Oxidation

DPN-cytochrome-b_5 reductase has now been obtained in a purified state and characterized as a flavin-adenine-dinucleotide enzyme with a molecular weight of 40,000 (41) and a redox potential (pH 7) $E_0 \simeq -330$ to -250 mv. Binding of DPN by this enzyme gives a new absorption maximum at 350 mμ (42). This enzyme can be active as a transhydrogenase, as demon-

strated by the reduction of acetyl-pyridine nucleotide by DPNH. One specific -SH group is involved in the binding.

Some results relevant to the intracellular distribution of cytochrome b_5 have recently been reported. Thus, from total liver and from isolated mitochondria, "cytochrome 556" and the related DPNH cytochrome reductase have been isolated (453, 454). These preparations could be identified with the known cytochrome-b_5 and DPNH-cytochrome reductase of microsomes. In view of only low contamination by microsomes, cytochrome-b_5 has been considered a component of mitochondria at an about equal level as the other cytochromes. This system is thought to be identical with the external, non-phosphorylating pathway of DPNH oxidation of mitochondria. The crystallisation of a cytochrome-b_5 preparation, which was purified on diethylaminoethyl cellulose, has been recently reported (455). The hemin pigments of intact microsomes have been investigated by difference spectrophotometry (456). A new absorption band at 450 mμ was obtained on addition of carbon monoxide to microsomes that had been treated with DPNH or dithionite.

The nature of the compound responsible for the band as 450 mμ is unknown. It is very sensitive to destruction of the microsomal structure by cholate and small pH shifts. The molar extinction of cytochrome-b_5 in intact microsomes could be determined by titration with DPNH and ferricyanide at $-6°$, thus eliminating auto-oxidation effects (456). This also shows that besides cytochrome-b_5 no other final electron acceptor is present in appreciable amounts. An attempted purification of the DPNH-cytochrome-c reductase complex may be cited (457, 458). Cytochrome-b_5 has also been isolated from microsomes of adrenal cortex (459). The DPNH oxidation by oxygen of these microsomes was shown to be increased by ascorbic acid (460). This reaction is assumed to be essential for hydroxylation of steroids in the adrenal cortex (461, 462).

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